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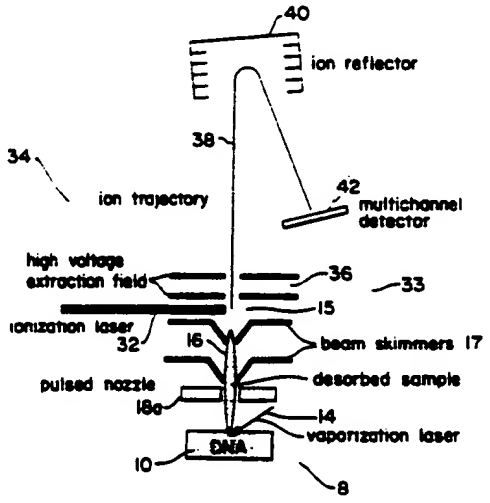

(57) Abstract

Described is a method and apparatus for analyzing an organic sample. In the preferred embodiment, this method and apparatus allows the determination of the base sequence of a nucleic acid by determining the molecular weights of the components of a biological sample. The method uses either a pre-existing chromophore or the covalent attachment of an ionizable chromophore t a biological sample followed by the vaporization of these molecules by exposure t an intense pulse of electromagnetic radiation (14) in the presence of a matrix which strongly absorbs the radiation. The gaseous molecules are subsequently extracted into an evacuated ionization chamber (15) and then exposed to electromagnetic radiation (32) at a wavelength which specifically excites the chromophore covalently attached to the biological sample.

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A METHOD FOR ANALYZING AN ORGANIC SAMPLE

Technical Field

The present invention relates, in general, to a method capable of identifying or determining the molecular weights of organic samples and, in particular, relates to the use of this method to construct an automated apparatus that can determine the sequence of nucleic acids utilizing non-isotopic and non-electrophoretic techniques.

Background Art

An important property of biological samples which often must be determined is their molecular weight. The most common method used to perform this measurement is to electrophorese the biomolecule through an acrylamide or agarose gel, visualize the position in the gel by staining or autoradiography, and determine the sizes by comparison to molecular weight standards of known sizes.

A related technology which uses similar sizing and detection techniques is DNA or RNA sequencing. DNA is a long thread-like macromolecule comprised of a chain of four deoxyribonucleotides which contain one of the four nitrogenous bases adenine (A), cytidine (C), guanine (G), or thymine (T). Similarly, RNA is composed of a long chain of ribonucleotides. The order of these nucleotides is the genetic code of the organism from which the DNA was isolated. The determination of this order is, therefore, a most important goal for scientists working in biological fields.

Manual meth ds to s quence DNA involv either synthesis of new DNA in the presenc of dide xyribo-

nucleotide terminators using as a template the DNA whose sequence needs to be determined or the degradation of the DNA to be sequenced using base-specific chemical treatments. In each case, a nested set of radioactively labelled DNA fragments are generated which represent the sequence of the DNA. For example, in the dideoxy method (Sanger, F. et al., (1977) PROC. NATL. ACAD. SCI. U.S.A., 74, 5463-5467), the template DNA, whose sequence is to be determined, is incubated with an oligonucleotide primer, four deoxyribonucleoside 5'-triphosphates (dATP, dCTP, dGTP and dTTP), and a DNA polymerase. The primer anneals to a specific complementary position in the template DNA that is defined by the order of the bases in the primer. The DNA polymerase then begins to catalyze DNA synthesis in the 5' to 3' direction by incorporating the deoxyribonucleoside 5'-triphosphate that is complementary to the next base in the template DNA. A complementary nucleotide is defined as one that follows the base-paring rules which require an A of one strand of a double-stranded DNA molecule always pairs with a T of the other strand and that a C of one strand always pairs with a G of the other strand.

In addition to the ability of DNA polymerases to incorporate normal nucleotides into the newly synthesized strand, many polymerases can also incorporate dideoxyribonucleoside 5'-triphosphates. Dideoxyribonucleotides are identical to deoxyribonucleotides except that they lack the 3'-hydroxy group on the ribose sugar. When these nucleotide analogs are incorporated into a growing DNA chain, synthesis terminates because the chain no longer bears the 3'-hydroxyl needed to add subsequent nucleotides. In the dideoxy sequencing method, four separate sequencing reactions are run, each containing one of the four dideoxyribonucleotides (each reaction also contains the four normal deoxyribonucleotides, one labeled with ^{32}P or ^{35}S). Incorporation of

the dideoxy analogs occurs occasionally and randomly in place of a normal nucl otid at complementary positions in the t mplat s that each reaction g nerates a heterogeneous population of product DNA molecules each beginning with the primer (and thus sharing a common 5'-terminus) and each terminating with the dideoxynucleotide that was included in that reaction.

The radioactively labeled products from each of the four dideoxy sequencing reactions are denatured to separate the newly synthesized DNA from the template and then electrophoresed in adjacent lanes on a polyacrylamide gel such that the DNA product molecules are separated based on their chain length. The presence of a band in the gel represents the presence of the corresponding complementary nucleotide in the template at a specific distance from the primer. Comparison and analysis of the bands present in each of the four lanes allows the sequence of the template DNA to be deduced. Thus, the relative positions of the bands identify the positions in the DNA sequence of each given nucleotide base. Generally, the DNA molecules are labelled so that the bands produced are readily detected. As shown in Figure 1, the intensity of the bands is generally non-uniform, within a single lane, because band intensity is directly related to the total number or concentration of DNA molecules of the same molecular weight in a specific lane, and this number varies from one molecule to another even when they are of approximately the same molecular weight and even when they contain the same chain terminating agent. Tabor and Richardson, U.S. Patent 4,962,020, have recently described a method for producing uniform banding, by use of a manganese buffer.

In the alternative chemical DNA sequencing method, ch micals that effect random partial cleavage of the DNA at G, G+A, C+T, and C ar added in four individual reactions to a single-stranded DNA fragm nt contain-

ing a ^{32}P label at the 5' end. The resulting fragments are processed as in the dideoxy method to d termine the DNA sequence. Maxam, A. and Gilbert, W. (1977) PROC. NATL. ACAD. SCI. U.S.A., 74, 560-564.

Automated DNA sequencing instruments based on the dideoxy method are described in U.S. Patent Nos. 4,855,255 and 4,811,218 and Prober et al., Science 16 Oct. 1987, 238; pp. 336-341. Both of these systems require the incorporation of four fluorescent dyes into the dideoxy-terminated product DNA which are then run on a polyacrylamide gel. The discrete-length product molecules are detected near the bottom of the gel by their emitted florescence following excitation with a laser. In these automated systems, many more sequences can be analyzed per gel and the sequences determined accurately out to 500 bases or greater. Furthermore, data can be recorded faster since there is no manual gel reading step required. Finally, the automated sequencers use non-isotopic detection methods so there is not added costs associated with radioactive wasted disposal.

Fluorescent labels can be used in place of radioactive labels, as described in Fung et al., U.S. Patent 4,855,225 and Hunkapiller et al. 4,811,218, and Prober et al., 238 Science 336, 1987. In addition, the DNA molecules may be labeled with different isotopic variants of an atom, e.g., sulfur. The sulfur atom is used as a marker for the specific nucleotide at the end of each nucleic acid molecule, and later identified by combustion of the molecule to produce sulfur dioxide, which is then detected using mass spectrometry. Brennan, U.S. Patent No. 5,003,059; Jacobson et al., U.S. Patent 5,002,868; and Serge, EPA 0 360 676 A1.

Although these instruments offer some advantages over manual methods, th y still suff r from numerous drawbacks which are inherent in the use f a polyacrylamide gel to resolv the DNA fragments. For

exampl , this method r mains labor-intensive since a gel must be poured and disp sed of for each sequencing run. Also, the accuracy of the sequencing can b impacted by artifacts generated by non-uniform gel matrix or even by a particular sequence as it electrophoreses down the gel. Furthermore, although more sequences can be determined on one gel that can be done manually, 10 to 12 hours are still required to obtain this data.

These problems associated with sequencing are minor when one is considering the generation of the sequence of a small genome, but they become monumental when contemplating sequencing the human genome, estimated to contain over 3 billion base pairs.

Mass spectral methods are well known. Pulsed mass spectroscopic methods, Burlingame, A.L. et al., (1990) ANAL. CHEM., 62, 268R-303R (and references therein), such as time-of-flight (TOF) and Fourier transform ion-cyclotron-resonance mass spectroscopy (FTICR-MS), have the inherent ability to simultaneously analyze all of the components of a complex mixture in a single 200 millisecond experiment. The most significant feature of a mass spectroscopic-based method is that it does not require prior electrophoretic or chromatographic separation prior to analysis thus reducing the analysis time by at least three orders of magnitude.

A major obstacle, until now, for implementing mass spectroscopy for analysis of large biomolecules has been the lack of an appropriate interface between the water-based biological system and the high vacuum required for mass analysis. Prior studies have used techniques such as secondary ion mass spectroscopy, Aberth, W. et al., (1982) ANAL. CHEM., 54, 2029-2034, fast atom bombardment, Griffen, D. et al., (1989) BIOMED. & ENV. MASS SPECTROM., 17, 105, ^{252}Cf plasma desorption, Sundqvist, B. et al., (1985) MASS SPECTROM, REV., 4, 421-460, el ctrospray, Fenn, J.B. et al., (1990) MASS SPECTROM

REVIEWS, 9, 37-70, and th rmospray, Straub, K. t al., (1990) RAPID COMMUN. MASS SPECTROM, 4, 267-271, Pramanik, B.C. et al., (1989) ANAL. BIOCHEM., 176, 269-277, in an attempt to transport biomolecules from the solid phase to the gas phase. These methods suffer either from severe sample decomposition or multiple charging problems. Other obstacles for mass spectral DNA sequencing methods include: guaranteeing inadequate mass resolution at 30,000-200,000 AMU (100-500 base strands); accomplishing selective and efficient ionization of DNA strands; and avoiding multiple ionization and/or fragmentation of DNA strands.

Laser vaporization may be used for the desorption of biological molecules into the gas phase, Karas, M. et al., (1989) BIOMED. & ENV. MASS SPECTROM., 18, 841-843. Proteins with molecular weight approaching 175,000 daltons have been molecularly desorbed with this technique and detected using TOF methods, Karas, M. et al., (1989) BIOMED. & ENV. MASS SPECTROM., 18, 841-843. Recently, Cotter et al., (1990) RAPID COMMUN. MASS SPECTROM., 4, 99-102 have demonstrated matrix-assisted laser vaporization and high resolution TOF detection of oligodeoxyribonucleotides with mass up to 1797 Dalton (6 bases). In this case, the positive molecular ion peak was intense with no apparent strand cleavage. Autoradiographic studies by Williams et al. suggest that extremely long DNA strands, containing up to 1,200 nucleotides, (1989) SCIENCE, 246, 1585-1587, can be transported into the gas phase intact.

Resonance-enhanced multiphoton ionization (REMPI), has been used to ionize many different biomolecules, including nucleotides and nucleosides, Li, L. et al., (1989) INT. JOURNAL OF MASS SPEC. & ION PROCESSES, 88, 197-210, peptides, amino acids, Grotemyer, J. et al., (1987) INT. J. MASS SPECTROM. ION PROCESSES, 78, 69-83, hormones, catecholamines, Pang, H.M. et al., (1988) APPL.

SPECTROSCOPY, 42, 1200-1206, and purines, Li, L. t al., (1989) INT. JOURNAL OF MASS SPEC. & ION PROCESSES, 88, 197-210.

TOF mass spectrometry has detected proteins with masses approaching 175,000 AMU, Karas, M., Ingendoh, A., Bahr, U., Hillenkamp, F. (1989) BIOMED. & ENV. MASS SPECTROM., 18, 841-843. This would correspond to a DNA strand of approximately 530 bases long. Finally, the extremely high sensitivity a TOF mass spectrometer allows the detection of ultra-low sample amounts in the sub-attomole range.

The difficulties of the prior art are overcome by the methods described herein to analyze an organic sample and/or to determine the base sequence of a nucleic acid.

It is an object of the present invention to use current sequencing technology with a mass spectral method to directly analyze the products of enzymatic DNA sequencing reactions.

It is the object of the present invention to solve inherent problems of the prior art described above using a combination of following techniques: (i) laser vaporization methods to desorb the liquid phase DNA strands into the gas phase; (ii) pulsed molecular beam nozzle techniques to transport the gas phase strands from a flowing helium atmosphere into the vacuum system; (iii) laser ionization methods to resonantly ionize a "tag" molecule on each DNA strand; and (iv) time-of-flight methods for high mass analysis.

It is an object of this invention to desorb biomolecules by mixing the sample of interest in an excess of a "matrix," or chromophore, which is specifically chosen to absorb light where the biomolecule does not. The chromophore absorbs the extremely high powered light (10^6-10^9 watts) that is presented in the short las r pulse (5ns).

It is an object of this invention to use this energy which is deposited in a short time so that all of the matrix and biomolecules are transported into the gas phase before thermal equilibrium can be attained.

It is an object of the present invention to place a single positive charge on the vaporized molecules using a technique called resonance-enhanced multiphoton ionization (REMPI). REMPI has been shown to be a very powerful tool for the analytical study of biological materials. The technique is based on selectively exciting an atom or molecule with a laser through specific vibronic states until the ionization energy is surpassed (Fig.2).

It is an object of the present invention to place on each vaporized DNA molecule a single charge by selective ionization of a covalently attached chromophore or "tag." These charged ions are then detected using time-of-flight (TOF) mass spectrometry.

It is an object of the present invention to use the combination of a solution-phase laser vaporization method with the ability to measure high masses using a TOF mass spectrometer to provide a rapid (<5 sec) method to completely analyze all of the nested strands produced from a given enzymatic dideoxy sequencing reaction.

It is a further object of the present invention to determine the sequences of the bases of a nucleic acid sample. Prior techniques are extremely slow and are highly labor intensive.

It is also a further object to describe an improved apparatus for passing tagged biological samples from a vaporizing source to an apparatus which would permit the detection of the sequences of the components of a biological sample such as a nucleic acid such as DNA or RNA.

Summary Of The Invention

Described is a method of analyzing an organic sample, comprising the steps of:

a) providing an organic sample in a medium which absorbs visible light;

b) vaporizing the sample and the medium into the gaseous state by subjecting the sample and the medium to electromagnetic radiation from an optical source wherein the vaporizing of the organic sample occurs in a visible light absorbing medium which absorbs the electromagnetic radiation in the visible light region;

c) ionizing the vaporized sample; and

d) detecting the contents of the vaporized, ionized sample.

An apparatus is described for accomplishing this analysis comprising an optical source for generating electromagnetic radiation in the visible light region for the purpose of vaporizing the organic sample present in a visible light absorbing medium, an ionization chamber containing an optical source in which the vaporized sample is exposed to electromagnetic radiation that excites the sample and ionizes the sample, and a means to detect the ionized sample to analyze the sample. Most preferably, a mass spectrometer will be used to determine the molecular weights of the ionized sample. In the preferred embodiment, the mass spectrometer is of the time-of-flight type.

To use this apparatus for sequencing DNA or RNA, the sample to be sequenced is used as a template according to the dideoxy sequencing procedure employing in these enzymatic reactions either primers or dideoxyrib nucleoside 5'-triphosphates that ar covalently attach d to a chromophore. The four nzymatic reactions products are then subj ct d individually to vap riza-

tion, ionization and mass analysis and the data correlated fr m each set t generate the sequenc for each nucleic acid sample.

Applicants have discovered that high molecular weight nucleic acid molecules can be vaporized without fragmentation. In general, this vaporization is performed by use of a high energy visible laser light in conjunction with a high concentration of a matrix which absorbs the laser light energy. The nucleic acid molecule to be analyzed is mixed with the matrix prior to vaporization. When the laser light illuminates the matrix, the matrix is vaporized and any nucleic acid molecule within the matrix is entrained, i.e., simultaneously vaporized, along with the matrix. By selection of a suitable matrix, wavelength of laser light, and laser energy level, it is possible to ensure that little or no fragmentation of the nucleic acid molecule occurs. The matrix and wavelength of laser light should be such that the laser light is absorbed by the matrix but not absorbed by the nucleic acid molecule, and chemical bonds within the nucleic acid molecule are not cleaved. Applicants have found that use of high laser levels (above 80 mJ/cm^2, or even 300 mJ/cm^2) for vaporization produces significantly improved results than use of a lower laser energy.

Brief Description Of The Drawings

FIGURE 1 is a diagrammatic representation of a dideoxy sequencing method;

FIGURE 2 shows a schematic view of 2-photon resonance enhanced ionization (REMPI) of a molecule M;

FIGURE 3 is a schematic representation of an apparatus of the present invention;

FIGURE 4 is a schematic r pres ntation of a vaporization chamber f the apparatus in Figur 3;

FIGURE 5 is a schematic representation of an optical system for vaporization;

FIGURE 6 is an optical system for REMPI;

FIGURE 7 is a side view of an apparatus of the present invention;

FIGURE 8 is a top view of the apparatus of Figure 7;

FIGURE 9 is a spectrum of analyzed samples by use of the apparatus of Figures 7 and 8;

FIGURES 10 and 11 are copies of autoradiographs showing a vaporized oligonucleotide;

FIGURE 12 is a graphical representation of the effect of laser energy on stability of dATP;

FIGURES 13A and 13B are copies of autoradiographs showing the effect of laser energy on populations of DNA molecules; and

FIGURE 14 is a diagrammatic representation of mock data from a mass spectrometer of the present invention, and its analysis to determine a nucleotide sequence. A, T, C and G represent the data obtained from populations of molecules having variable ends terminating in the corresponding nucleotide.

Detailed Description Of The Invention

This invention includes a method for analyzing and identifying the components of an organic sample and an apparatus for accomplishing this method. In the preferred embodiment, the identification is through a determination of the molecular weights of these components and the preferred components are the products of a dideoxy sequencing reaction.

This discovery allows the determination of molecular weights of nucleic acid molecules in a solution or in a solid state, and for the determination of the molecular weights of several different nucleic acid

molecules within a mixture of nucleic acid m lecules. To this nd, th inventi n is particularly useful for determination of the nucleotide sequence of DNA or RNA, without the need for any separation of nucleic acid molecules generated in a sequencing reaction.

For example, a population (series) of nucleic acid molecules can be formed by use of standard techniques, such as those described above, and directly analyzed by causing vaporization of each of the molecules and determination of their molecular weights. If four such populations of molecules, ending in A, T, G, or C, respectively, are created, the nucleotide sequence can be determined by comparing the molecular weights of molecules within each of the four populations. Such comparisons can be performed by use of a computer, and allow analysis of a large number of sequencing mixtures within a very short time.

Because analysis by mass spectrometry does not require separation of nucleic acid molecules from one another to obtain molecular weight, the methods of this invention provide significant advantages over prior sequencing methods which rely, in general, on use of polyacrylamide gel electrophoresis to separate the molecules. Thus, the sequencing methods described herein eliminate the most labor-intensive and time-consuming steps of DNA sequencing methods.

It is also possible, because of the enhanced sensitivity of this method, to substantially reduce the amount of reagents needed for sequencing. For example, sequencing can be performed with a reduced amount of a polynucleotide template and enzyme used in a dideoxy sequencing reaction. Applicants' method also provides significant time savings because mass spectrometry det rminations can be made quickly. In addition, the t chnology d scribed h r in can be easily automat d for r p ated sample analysis. Thus, th s quencing m thods

describ d herein have many advantages over currently available methods.

In general, a m thod of this invention features vaporization of a standard DNA sequencing solution containing nucleic acid molecules of varying molecular weights, modification of the vaporized molecules so that they are susceptible to analysis by mass spectrometry, for example, by ionization, and performance of mass spectrometry on the vaporized and ionized molecules.

Thus, in a first aspect, the invention features a method for analyzing a nucleic acid molecule, without fragmenting the molecule, by vaporizing a mixture of the molecule and a matrix by illuminating the mixture with visible laser light absorbed by the matrix and not by the nucleic acid molecule.

By "vaporizing" is meant that the nucleic acid molecule is caused to enter the vapor phase such that it is available for analysis by a mass spectrometer, or available for ionization and subsequent analysis by a mass spectrometer.

By "matrix" is meant any component of a mixture with the nucleic acid molecule which is adapted to absorb visible laser light, and can be vaporized by that laser light, and is adapted to simultaneously cause vaporization of the adjacent nucleic acid molecule. That is, the vaporized matrix entrains the embedded nucleic acid molecule and carries it into the vapor phase. Generally, this matrix is vaporized by visible laser light having a wavelength between about 400 and 1100 nanometers, preferably between 500 and 550 nanometers, and most especially 532 nanometers, which is not absorbed by the nucleic acid molecule.

In a related aspect, the invention features a m thod for determining the nucl otide s quence of a polynucl otide by using mass spectrometry to determin th molecular weights of individual single-strand d

nucleic acid molecules in a population. The population includes a plurality of single-stranded nucleic acid molecules generated from the polynucleotide (e.g., by a sequencing technique described above), each nucleic acid molecule having a different molecular weight and one defined terminus and one variable terminus which terminates at a specific nucleotide. Generally, this method is performed without prior separation of the nucleic acid molecules from each other.

By "mass spectrometry" is meant any technique which allows the molecular weight of a nucleic acid molecule in the vapor phase to be determined. Those of ordinary skill in the art will recognize that many specialized apparatus, generally termed mass spectrometers, are known which are specifically adapted to perform the technique of mass spectrometry, e.g., a time-of-flight mass spectrometer which is particularly suited to detection of molecular weight of large molecules.

As discussed above, methods for generating nucleic acid molecules from a polynucleotide to determine nucleotide sequences are well known in the art. For example, such techniques include the use of a chain termination agent in a technique generally called dideoxy chain termination sequencing (see, Sanger et al., supra, and Tabor and Richardson U.S. Patent No. 4,795,699), or the technique generally described by Maxam and Gilber, supra. It is particularly important in the sequencing method that molecules of one molecular weight be provided in approximately equal numbers to molecules of similar molecular weight as, for example, described by Tabor and Richardson, U.S. Patent No. 4,962,020. Thus, the population includes several sets of nucleic acid molecules of identical molecular weights, each set having a different molecular weight from each other set.

Each of the nucleic acid molecules in the population has a defined terminus, that is, each molecule has an identical 3' terminus or 5' terminus containing a chain of identical nucleotides. Each molecule also has a variable terminus, that is, the other of the 3' or 5' terminus is different for each set of nucleic acid molecules within the population. As will be recognized by those or ordinary skill in the art, for each molecule in the population, the variable terminus generally ends in a specific identical nucleotide.

In preferred embodiments of the above aspects, the determining step includes vaporizing a mixture of matrix and the population of nucleic acid molecules derived from the polynucleotide to be analyzed. The mixture is vaporized by illuminating it with visible laser light absorbed by the matrix. As discussed above, the nucleic acid molecules are generated by chemical degradation of the polynucleotide, or by extension of a short polynucleotide, called a primer, complementary to a portion of the polynucleotide to be sequenced by a DNA polymerase (e.g., T7 DNA polymerase) in the presence of a chain terminating agent, e.g., a dideoxynucleoside triphosphate, preferably in the presence of manganese. The polynucleotide may be a DNA or RNA molecule.

In other preferred embodiments, the determining step includes comparing the molecular weight of the nucleic acid molecules, to provide a nucleotide sequence, e.g., by use of a computer; the vaporizing step is performed using a laser adapted to emit a pulse of light, e.g., with a power greater than about 80 mJ/cm^2, or preferably greater than about 120 mJ/cm^2, or most preferably greater than about 320 mJ/cm^2; the laser is a neodium yttrium aluminum garnet laser; and the pulse of light is less that 10 nanoseconds, and preferably about 5 nan seconds.

In yet other preferred embodiments, the matrix is an organic dye, e.g., rhodamine 6G, with a ratio of matrix weight to total nucleic acid molecule weight ranging from about 1:1 to 100,000:1, more preferably 1,000:1 to 25,000:1; the matrix may also be chosen from Rhodamine 6G, Rhomdamine 700 or 800, DTTCI, LC8800, DNTTCI, HDITCI, DDCI4, and dibenzocyanine 45 or from any one of a number of chromophores which absorb light between 400-1100 nm; and each nucleic acid molecule is bonded to an ionizable chromophore which allows ionization of each molecule, e.g., a fluorescent dye, selected from the group including fluorescein, rhodamine, tetramethylrhodamine, sulforhodamine 101, nitrobenzo-2-oxa-1-diazaole, anthracene, pyrene, coumarin, acridone, N-5-dimethyl amino naphthene, and derivatives thereof (including iodoacetamide, maleimide, isothiocyanate and succinimidyl carboxylate) and the like. Most preferably, the chromophore absorbs light of a wavelength greater than 300 nanometers, and is connected to the molecules by covalent bonding or by a linker arm (see e.g., Hunkapiller et al., U.S. Patent 4,811,218 and Fung et al., U.S. Patent 4,855,225), and preferably positioned between 1 and 50 atoms from the molecule.

Other preferred embodiments further include ionizing the nucleic acid molecules, after the vaporizing step, and determining the molecular weight of the vaporized ionized nucleic acid molecules using a mass spectrometer, e.g., a time-of-flight spectrometer. Preferably, a single positive charge is placed on each vaporized nucleic acid molecule prior to analysis by mass spectrometry. One technique for placing such a single positive charge is by resonance-enhanced multiphoton ionization (REMPI).

In another related aspect, the invention features an apparatus for determining the nucleotide sequence of a polynucleotide. The apparatus includes a

mass spectrometer adapted to determine the m lecular weight of individual single-stranded nucleic acid molecules (derived from th polynucleotide) in a first plurality of different populations (e.g., four separate A, T, C, and G populations), each population including a second plurality of different single-stranded molecules, each having a different molecular weight and one defined terminus and one variable terminus, the variable terminus terminating at a specific nucleotide, with the variable terminus of each first plurality of different populations terminating at a specific nucleotide. Also provided is a computer adapted to compare the molecular weights of each of the molecules in the populations to provide the nucleotide sequence of the polynucleotide.

In a further related aspect, the invention features one or more populations of vaporized and ionized nucleic acid molecules including a plurality of different single-stranded nucleic acid molecules each having a different molecular weight and one defined terminus and one variable terminus, the variable terminus terminating at a specific nucleotide.

A feature of the invention, which uses laser desorption of biomolecules, is to mix the sample of interest into an excess of a "matrix" or chromophore, which is specifically chosen to absorb light where the biomolecule does not. It is believed that the chromophore absorbs the extremely high-powered light (10^6 - 10^9 watts) that is present in the short laser probe (5 ns). Possible chromophores includes nicotinic acid, sinapinc acid, ferulic acid, nitrobenzyl alcohol, benzene, diiodomethane, rhodamine G, and the like. This energy is deposited in such a short time that probably all of the matrix biomolecules are transported into the gas phase before thermal equilibrium can be attain d. It has been shown that littl or n degradation of thermal-

ly labile molecules results from this desorption technique.

If the sample does not contain a suitable chromophore for resonance enhanced multiphoton ionization, then a light-absorbing chromophore may be covalently linked to the sample. There are a large number of dye moieties which are appropriate for this ionization process. Suitable dyes include fluorescein and fluorescein derivatives, rhodamine and its derivatives, tetramethylrhodamine and its derivatives, sulforhodamine 101 (Texas red) and its derivatives, nitrobenzo-2-oxa-1-diazole, anthracene, pyrene, coumarin, acridone, N-5-dimethyl amino naphthene, and their derivatives and the like. Derivatives of each of these dyes are commercially available from (Molecular Probes, Inc., Eugene, Or) in forms that can be easily linked to appropriately activated biological or chemical samples (e.g., containing an available amine or thiol group). The most appropriate of the available derivatives of these dyes for the purpose of linking to biomolecules include: iodoacetamide, maleimide, isothiocyanate, and succinimidylcarboxylate and the like. The appropriate functionality on the biomolecule to link to the first two in this series is SH, while the latter two require an NH_2 group present on the biological or chemical sample. For a listing of suitable dyes, see U.S. Patent No. 4,821,218 and 4,855,225, hereby incorporated by reference.

There are a wide variety of standard procedures for chemically binding these types of chromophores to organic and biological molecules (see, for example, U.S. Patent Nos. 4,821,218 and 4,855,225). Amine and thiol-containing nucleic acids can also be prepared and used to link to th appropriate r active group n the dyes. Oligonucleotides are commercially availabl fr m many sourc s that contain either a reactiv amine or

thiol group at the 5' end (Clonetech, Palo Alt , CA). These are linked to the corresponding reactive groups on the dyes using standard procedures. There are also a number of commercially available oligonucleotides which are coupled to fluorescent dyes. For example, ABI (Foster City, CA) sells four oligonucleotides that are used in their automated DNA sequencer that are covalently linked to two fluorescein (fluorescein and 2', 7'-dimethoxy-4,5-dichlorofluorescein) and two rhodamine (tetramethylrhodamine and rhodamine X) derivatives.

These same dyes can also be covalently linked to proteins allowing detection and molecular weight determination of protein mixtures in biological samples. Unlike the nucleic acid modifications, proteins contain many reactive functional groups which will react with the preferred linking groups of the dye derivatives (isothiocyanate, succinimidylcarboxylate, iodoacetamide, and maleimide). For example, iodoacetates react predominantly with SH groups of free cysteines but may also react, if cysteines are absent, with methionines, histidines, or tyrosines. Maleimides are also primarily thiol-reactive but will also react with a amines at higher pH. Since many proteins do not have free thiols, the amine reactive functional groups, isothiocyanates and succinimidylcarboxylate, which react with free aliphatic amines are often the best choice for these types of coupling. Essentially, all known proteins have lysines and most have a free amino terminus. Therefore, these amine reactive dye derivatives are often the reagents of choice for protein modifications.

Vaporization Chromophore (Matrix)

It is important in the invention to mix the nucleic acid molecules to be analyzed with an excess of a matrix which is specifically chosen to absorb light

energy at a wavelength not absorbed by the molecules. It is believed that the matrix abs rbs the extremely high-powered light that is present in a short laser pulse (less than 10 nanoseconds (ns), most preferably less than 5 ns), and is thereby vaporized. Examples of such matrices include Rhodamine 6G, Rhodamine 700 or 800, DTTCI, LC8800, DNTTCI, HDITCI, DDCI-4, and dibenzocyanine 45. Applicants believe that, because the laser energy is received by the sample over only a short time, all of the nucleic acid molecules within the matrix are caused to enter the vapor phase by entrainment before any fragmentation of the nucleic acid molecules can occur.

Ionization Chromophore

The present invention provides laser vaporization methods to desorb liquid phase nucleic acid molecules into the gas phase, and laser ionization methods to resonantly ionize each nucleic acid molecule. Unmodified nucleic acid molecules are not readily ionized at wavelengths above 300 nm. Thus, an ionizable group is introduced onto each molecule. For example, a single chromophore is introduced into each nucleic acid molecule so that a single positive charge an be introduced on each vaporized nucleic acid molecule by resonance-enhanced multiphoton ionization (REMPI). For example, referring to Figure 2, an uncharged nucleic acid molecule (M) is raised to an excited electronic state (M*) by a first photon, and then to an ionized state (M+) by a second photon. These ions (M+) can then be detected using time-of-flight (TOF) mass spectrometry.

Suitable chromophor s or dyes are recited abov .

It is desirable that the chromophore is positioned at least 1 at m and possibly up t 50 atoms away fr m the nucleic acid molecules strands by us of a linker arm of appropriate lengths.

The chromophores can also be covalently linked to a nucleotide. For example, when used for DNA sequencing, these nucleotides can be used as the four dideoxyribonucleotides containing the four bases A, C, G, and T. E.I. DuPont (Wilmington, DE.) sells the four dideoxynucleotides covalently attached to four different fluorescein dyes. ABI sells four dideoxynucleotides covalently attached to four rhodamine chromophores. Also, there are several standard procedures by which a dideoxynucleotide can be coupled to a chromophore. For example, synthesis of the dideoxy-TTP derivative can be accomplished by converting dideoxy-UTP (ddUTP) to 5-(3-amino)allyl ddUTP by the method described by Langer et al. 78 PROC. NATL. ACAD. SCI. U.S.A., 6633, 1981. The cytidine analog can be formed by converting dideoxyuridine to the 4-hexylamine derivative using a procedure similar to that described by Horn et al., 17 NUCLEIC ACIDS RES., 6959, 1989, coupling to the NHS ester of a selected chromophore, followed by conversion to the triphosphate by one of several methods, e.g., as described by Kozarich et al., 12 BIOCHEMISTRY, 4458, and Ruth et al., 20 MOL. PHARMOCOL., 415, 1981. The dideoxyadenosine or guanosine derivatives can be similarly prepared by lithiation of the purine ring at C8 followed by alkylation with a suitably protected amine-containing alkylhalide Barton et al., TETRAHEDRON LETT., 279, 1979. Alternatively, the adenosine derivative can be prepared by iodination at C7 of the adenosine derivative, dideoxytubercidin, followed by coupling to N-trifluoroacetylpr pargylamin und r Pd (0) catalysis. Robins et al., 48 J. ORG. CHEM., 1854, 1983.

It is important that only one chromophore be attached to each nucleic acid molecule to ensure a single positive charge on each molecule after ionization. The chromophore procedures discussed above enable detection of the molecules by allowing the placement of precisely one unit of positive charge per nucleic acid molecule, permitting an exact determination of molecular weight. Single ionization of each molecule greatly simplifies the appearance of a mass spectrum of mixtures of nucleic acid molecules.

It is desirable that the chromophore absorb at a wavelength greater than 300 nm (where DNA weakly absorbs). In addition, the chromophore should have excited states which allow resonant ionization. In one example, when an anthracene chromophore is used, it is believed that the solution phase electron excitations may be centered around approximately 380 nm, 320 nm, and 280 nm. Thus, an ionization scheme as shown in Figure 2 is possible.

Apparatus

The apparatus of the present invention can be used to analyze biological or chemical samples generated from any one of a number of sources. For example, it is possible to analyze blood samples for the presence of various metabolites or proteins or even modified proteins. However, in the preferred embodiment, this instrument will be used to sequence DNA.

The DNA samples to be sequenced are processed according to the Sanger dideoxy sequencing method described above. These reactions are run with the light absorbing chromophor linked to either th primer or the dideoxyribonucleotides. . Each of the four enzymatic reactions containing th did oxy-terminated product DNA covalently linked t a light-absorbing chromophore are

then mixed with an excess (e.g., 10-100,000 fold by weight excess) of rhodamine 6G and each of the four mixtures are placed individually into sample holder 9. In the case where the sample is analyzed in the solid phase, the water is removed by evaporation. In the case where the sample is analyzed as a liquid, the sample holder is placed directly into the vaporization chamber 21. The sample is then exposed to a 1-100 MW pulse from the laser at 532 nm which vaporizes the rhodamine 6G, and via entrainment, the biological sample. This vaporized material is extracted through the pulsed nozzle 18a by the flowing helium stream, ionized by a 1 MW pulse from the eximer laser, and extracted by the three kilovolt potential applied to the 90% transmission grid into the time-of-mass spectrometer. The molecular weights of the ions detected by the mass spectrometer are recorded. Then this entire process is repeated in sequence for the remaining three dideoxy sequencing reactions, the results from the four samples correlated, and the DNA sequence deduced as is done with manual or other automated sequencing methods.

Referring to Figure 3, a schematic representation of an apparatus of the invention is provided. Specifically, apparatus 8 includes a sample holder 10 in which the nucleic acid molecule of interest is placed within a matrix. This sample holder is positioned to allow electromagnetic radiation, e.g. laser light, from a vaporization laser 14 to contact the nucleic acid molecule. Also provided is a pulsed nozzle 18A which is positioned relative to sample holder 10 to allow vaporized matrix and nucleic acid molecule to pass in a series of pulses as a desorbed sample (shown generally at 16) through a pair of beam skimmers 17 into a location 15 at which the volatilized nucleic acid molecules can be ionized by electromagnetic radiation from an ionization laser 32. Such ionized and vaporized nucleic

acid molecules are then directed by use of a high voltage electric field 36 into a mass spectrometer (shown generally as 34) through an ion projectory 38 via an ion reflector 40 to a multichannel detector 42.

Referring to Figure 4, a detail of a vaporization chamber used in apparatus 8 (shown in Figure 3) is provided. This chamber includes the sample holder 10 on which aqueous sample 12 is deposited. Above this holder is a pulsed nozzle 18A which extracts gaseous sample resulting from vaporization of the sample by laser light from vaporization laser 14, together with the carrier gas, helium, which enters vaporization chamber 21 through inlets 19 and 19A. The carrier gas causes the vaporized sample to pass out of the vaporization chamber toward the laser beam of ionization laser 32 where the sample is ionized. A window 20 is provided in the upper portion of vaporization chamber 21, and a second transparent window 29 is provided in the lower portion of the chamber, to allow passage of laser light from vaporization laser light 14. The base of the pulse nozzle is shown generally by the numeral 26.

The vaporization chamber 21 shown in Figure 4 can be maintained at either vacuum or ambient pressure. While not specifically shown, a high efficiency pumping system is provided to evacuate the low pressure chambers of this apparatus. Generally, the vaporization chamber is maintained at ambient pressure. When the sample is a liquid, the sample holder is oriented in a horizontal plane so that a liquid sample will not drip or run, and is situated so that it can be exposed to electromagnetic radiation. The sample holder is constructed from either polished 305 stainless steel or glass and is removable. It is affixed so that the sample can be reproducibly positioned in precisely the same location with respect to the pulsed nozzle. Alternatively, the sample can be dried to a solid by evacuation.

The source of the electromagnetic radiation to cause vaporization is generally a laser, e.g., a neodinium yttrium aluminum garnet (Nd YAG) laser. This laser is operated in a mode which provides radiation at 532 nm up to 500 mJ/cm^2. The light is columinated to provide high enough power to effect vaporization of at least a portion of the sample. Figure 5 shows that the columinated light 14' from a laser is directed through an optical system. This system includes two pellin broca prisms (not shown) which remove unwanted frequencies of light to a beam dump for removal. The remaining light is then directed, first through an iris to restrict beam size, and then through focusing optics 50 constructed from a non-absorbing quartz S1 UV optical flat (1" x 1/8") into the vaporization cell to impinge onto the sample from above. The laser is operated in such a manner so as to produce an intense flash of electromagnetic radiation which will be absorbed by the vaporization matrix and not the sample.

Once vaporized, as discussed briefly above, the sample is ejected into a helium atmosphere for subsequent processing. The helium injection and exhaust ports are positioned to maximize the flow of vaporized product toward the pulsed nozzle. The flow of helium is maintained using a He flow meter as supplied from Tylan Corporation. The output of the helium flow is connected to a pump via a metering valve. The pulsed valve is commercially available from Thermionics Laboratory, beam dynamics. The pulsed valve is positioned between 1 and 10 mm above the surface of the sample to be vaporized. The pulsed valve is timed to open 0.1 to 100 microseconds after the firing of the vaporization laser. The sample is extracted from the vaporization chamber into the ionization chamber, and a potential simultaneously applied to a 905 transmissi n grid to extract any ions

which form during the vap rization process, and survive the transit through the pulsed n zzle.

Once the sample has entered the vacuum chamber, a second intense burst of electromagnetic radiation is delivered into the apparatus from an excimer or YAG laser source. In the preferred embodiment, the ionizing radiation is at 300-500 nm, most preferably 300-380, e.g., 345 nm. As shown in Figure 6, this beam is developed using optical components. The beam 32' is directed through a telescoping cylindrical lens 52 to shape the beam into a compact pulse. The beam is then passed through slits 54 to define the final shape of a ribbon of about 1 mm x 5 mm in size. The beam is then directed through a quartz window 56 into the vacuum chamber. In the vacuum chamber, the light intersects the beam of sample and matrix molecules at a 90 degree angle. The sample absorbs the radiation and the contained molecules ionized by resonant multiphoton processes.

The ionized sample is extracted by a three kilovolt potential applied to a 90% transmission grid (see Figure 3, field 36) which is 10 cm from the plane of the ionizing radiation. The ions formed in the electromagnetic pulse are then extracted into a mass spectrometer 34 (Figure 3), e.g., a time-of-flight mass spectrometer, such as a Bruker TOF 1 (Bruker Instruments, Inc. of Billerica, Mass.) This system operates at 30 Hz, with a very large sample depth (128K) and high resolution (16 bit). This system includes the required electronic controls and an ultra high vacuum pumping system that can be used for the ionization chamber.

Methods

The polynucl otid to be sequenc d is processed according to the Sanger dideoxy s qu ncing

reaction method described above, or any other sequencing method. These reactions are preferably run with a light absorbing chromophore linked to either the primer or the dideoxyribonucleotides. Each of the four enzymatic reactions containing the dideoxy-terminated molecule covalently linked to a light-absorbing chromophore is then mixed with an excess (e.g., 10-100,000 fold molar excess) of rhodamine 6G, and each of the four mixtures placed individually into the sample holder. When the sample is in the solid phase, the surrounding liquid medium, e.g. water, is removed by evaporation. When the sample is a liquid, the sample holder is placed directly into the vaporization chamber.

The sample is exposed to a 1-100 MW pulse from the laser at 532 nm which vaporizes the rhodamine 6G, and via entrainment, the nucleic acid molecule. This vaporized material is extracted through the pulsed nozzle by the helium stream, ionized by a 1 MW pulse from the excimer laser, and extracted by the three kilovolt potential applied to the 90% transmission grid into the time-of flight mass spectrometer. The molecular weights of the ions detected by the mass spectrometer are recorded. This process is then repeated in sequence for the remaining three dideoxy sequencing reactions, the results from the four samples are correlated, and the nucleotide sequence deduced. An example of such data is shown in Figure 14. Correlation of these data can be performed manually or by computer using a program which determines the relative molecular weights of each molecule in each population of molecules. Such a program is readily formulated by those skilled in the art.

Those skilled in the art will understand that there are many variations of the above apparatus and method which fall within the purview of this invention. For example, different matrices can be used for the

vaporization processes and different chromophores can be used for the ionization processes, as can different sources of electromagnetic radiation be used. The sample may require pre-treatment by various procedures to increase sensitivity levels, for example, removing the template polynucleotide, removing the substrate nucleotides, exchanging counterions, or removing any proteins prior to analysis. Furthermore, the sample holder and vaporization chamber can be modified so as to accept multiple samples by the addition of a movable stage that will bring each of the various samples into register for the vaporization step.

The "tag" molecule in this mass spectral technique will perform a role analogous to the fluorescent tag employed in other automated electrophoretic DNA sequencing techniques. In the mass spectral technique, the "tag" will enable the detection of the DNA components by allowing the placement of precisely one unit of positive charge per DNA strand, permitting an exact determination of molecular weight or strand length. Single ionization of each strand will greatly simplify the appearance of mass spectrum of mixtures of DNA strands.

For a desirable REMPI tag for mass spectral analysis of DNA strand, it is most desirable that the molecule have an adsorption band greater than 300 nm (where DNA weakly absorbs). Secondly, the molecule should have excited states which allow resonant ionization. Lastly, the molecule should be chemically attachable to the DNA strand during the enzymatic reaction. Utilizing a tetramethylrhodamine chromophore, it is believed that the solution phase electron excitations may be centered around approximately 350 nm, 250 nm, and 200 nm for similar compounds. It is also desirable that the tag dye molecule be positioned at least 8 atoms and possible up to 29 atoms away from the DNA strands,

depending upon the length f the linker arm that is chosen.

The i nized sample is th n extracted by a three kilovolt potential applied to a 95% transmission grid which is 10 cm from the plane of the ionizing radiation. The ions formed in the electromagnetic pulse are then extracted into a mass spectrometer. In the preferred embodiment, the mass analysis system is of the time-of-flight type, such as a Bruker TOF 1 (Bruker Instruments, Inc. of Billerica, Mass). This system has been developed to operate at 30 Hz, with a very large sample depth (128K) and high resolution (16 bit). This system is complete in terms of control electronics and also contains all of the ultra high vacuum pumping systems that will be required for the ionization chamber.

The following examples are illustrative of the invention. They were performed in the sequencing chamber shown in Figures 7 and 8. The chamber consists of a solid film vaporization system juxtaposed to a REMPI TOF chamber. With the vaporization system, a 2 μl spot of a mixture of sample and rhodamine 6G is placed on a glass microscope slide, the sample allowed to dry and the glass slide fixed to a stainless steel rod sample holder. The spot is irradiated with a laser pulse and the vapor plume travels toward the electrostatic grid plates of the time-of-flight mass spectrometer.

The lens system for the mass spectrometer is shown in Figure 7. Four grid plates (1-4) form the acceleration optics. The following is a description of the ion optical system starting from the leftmost grid plate shown in Figure 7. The first plate has a 1/8" aperture in its center. This plate is grounded for these examples. The next plate c ntains a 90% transmission grid which is typically bias d positive 1000 volts

with respect t ground. On function of this grid is to repel positively charged ions back t the ground plate so that only neutral molecules enter the ionization region of the mass spectrometer. The next plate is typically biased positively to 950 volts with respect to ground. The region between plates 2 and 3 is called the ionization region because the resonance-enhanced multi-photon ionization occurs here. This region also forms a low voltage extraction region for the dual slope acceleration scheme which serves to decrease the full-width-at-half-maximum of the signal peaks. The fourth plate contains an 82% transmission grid and is grounded. The region between plates 3 and 4 represents an acceler-ation of 950 eV per ion. The ions then enter a 63cm field free drift region of the mass spectrometer.

At the end of the mass spectrometer is a high molecular weight ion detector 68. This consists of one stage from a CuBe electron multiplier detector. This "venetian" blind ion conversion stage is biased to 10 keV to convert high molecular weight ions to smaller ions and electrons. This type of ion conversion system has the added advantage of protecting the multichannel ion detector from carbonaceous contamination. The resulting particles are then directed to the micro-channel plate detector for current amplification. The signal is amplified further in a fast ampli-fier/discriminator. The signal is then directed to a digital storage scope where current or signal is stored as a function of time. A plot of current versus time forms the time-of-flight mass spectrum.

A schematic of the entire sequencing system is shown in Figure 8. The vaporization chamber and TOF mass spectrometer are pumped by a turbo molecular vacuum pump. The laser beams n cessary for ionization and vaporization ent r through quartz windows. Th optical setup allows th beams to enter th chamber thr ugh the

sam window. The vaporization laser is directed at the front of the thin film system by a turning prism, mounted as shown in Figure 7. The ionization beam travels parallel to the plane of the extraction plates. The timing between the two laser pulses is maintained by precision delay circuits. The minimum vacuum for an experiment is approximately 1 x 10^{-4} torr.

The DNA sample mixture 74 was spotted onto a glass coverslip attached to the end of the stainless steel sample positioner. The mixture of laser dye and tagged DNA sample was allowed to dry into a solid thin film on the coverslip. The sample was then loaded into the vacuum chamber and pumped to a pressure of approximately 5 x 10^{-6} torr.

In both example 1 and 2 described below, the vaporization laser struck the thin film at an angle of approximately 45 degrees from the surface normal. The vaporization laser used was the second harmonic of a Nd YAG III laser, (532 nm, 6 ns pulse length, variable power). The YAG laser was equipped with gaussian optics so that the photon density within the beam was approximately constant across the diameter of the beam. The diameter of the vaporization laser beam was irised to 1 mm. The fluence of the beam ranged between 10 and 80 mJ/cm^2 s measured by a power meter (not shown). The sample positioning system was rotatable so that fresh sample could be continuously brought into the area of vaporization, if necessary.

A multiphoton ionization laser 80 was used to generate a 345-370 nm photon and 15 ns pulse length, which passed parallel to the plane of the thin film at a distance of 11 mm from the surface. The beam was irised and passed through 1 mm slits. The beam shape of this ionization laser was a ribbon having dimensions 1 mm x 7 mm. for the experiments requiring ionization f the DNA sample, th excimer laser was triggered to fire

at a time of 45 microseconds after the vaporization laser hit the target. Th puls of photons from th ionization laser was detected using a photodiode. The signal from the photodiode defined time = zero for the time-of-flight measurement.

Example 1

A sample of anthracene-labeled thymidine-5'-monotriphosphate was mixed at a 1 to 1 molar ratio with rhodamine 6G dye. The sample was then spotted onto the sample holder as described previously. The vaporization laser at 532 nm was directed onto the sample thin film with a fluence of 40 mJ/cm^2. The ionization laser at 345 nm was maintained at 30 mJ/cm^2. A representative time-of-flight spectrum prepared from the oscilloscope 86 connected to the multichannel detector inputs 68 and the photodiode 82 via lines 88 and 90, respectively, is shown in Figure 9.

In this plot (Figure 9), the current signal output from the multichannel detector was plotted as a function of time in microseconds. The peak appearing at 32.7 microseconds, labeled A, was identified as pure rhodamine 6G (MW = 479 AMU) by control experiments where no labeled nucleotide was present.

When the anthracene-labeled nucleotide sample was added to the thin film, the peak at 41 microseconds, labeled B, appeared. This arrival time implied that a species of approximately 650 AMU was present in the vaporized sample. Therefore, the mass calculated for the tagged nucleotide is 650 AMU.

Example 2

In this exampl , th ion d t cti n system f Figure 7 was removed fr m the apparatus and a piec of

filter paper installed to collect all of the laser vaporizati n materials. A 4 mm diameter hole was plac d in the filter paper to allow passage f th vap rizati n laser. The paper was positioned at a distance of 10 mm from the vaporization spot on the thin film.

An oligonucleotide (50 pmol) having the sequence 5'-GTTTTCCCAGTCACGAC-3' was synthesized, purified by high pressure liquid chromatography (HPLC), and labeled at the 5' end with ^{32}P using polynucleotide kinase. The labeled oligonucleotide was purified from unreacted ATP using a Waters Associates Sep-Pak C18 cartridge (Maniatis et al., Molecular Cloning, A Cloning Manual), Cold Spring Harbor Press, New York, 1982). The final specific activity of the oligonucleotide was 300 counts per minute per femtomole (cpm/fmol). Five picomoles (pmol) of the oligonucleotide was dissolved in 4 μl of water containing 10 mg/ml rhodamine 6G. This resulted in a final molar ratio of sample to matrix of 1:17,000. This mixture was then spotted in two 2 μl aliquots on a glass cover slip. The dried sample was placed in the sample chamber, the chamber evacuated to 5 x 10^{-6} torr, and then the sample was exposed to the second harmonic of the Nd YAG III laser (532 nm, 8 ns pulse length) at a power equal to 130 mJ/cm^2. The filter containing the putative vaporized DNA was removed and the process twice repeated on fresh samples at power levels equal to 85 and 45 mJ/cm^2, respectively. Each filter was then exposed to Kodak XAR-5 X-ray film to obtain the distributions shown in Figure 10: panel A, 130 mJ/cm^2; panel B, 85 mJ/cm^2; panel C, 45 mJ/cm^2.

Three features of the vaporization process are revealed by this analysis. First, as the laser power was increased, the amount of molecular vaporization product also increas d. Second, th images f the vaporiz d material on th filter paper revealed a highly dir ctional vaporization pr cess. The distribution is

peaked in th normal directi n and was considerably tighter than a simple c sine distributi n expect d for a thermal desorption process. Third, the ^{32}P pr sent on the filter paper was evenly distributed, as is expected for molecular vaporization. Spallation, or the removal of macroscopic pieces of the mixture, has been shown in prior studies (Nelson et al., SCIENCE 1585, 1989) to lead to a spotted or speckled appearance. Spallation features, were observed in our experiment when vaporization was performed at atmospheric pressure on a liquid sample. Taken together, the images of the distribution obtained in these experiments strongly suggest that individual molecules were being vaporized.

Example 3

To characterize the products obtained from the laser vaporization of the above 17-mer, the radioactive material on each filter (Example 2) was eluted by soaking in water, and each mixture then analyzed by polyacrylamide gel electrophoresis.

The autoradiographs of the filter papers shown in Figure 10 were used to indicate where the vaporized radioactive oligonucleotide was deposited onto the filters. These portions were excised and then extracted with two 150 μl portions of water. The resulting solutions were concentrated, and loaded onto a 20% polyacrylamide gel: (Figure 11) lane 1, 130 mJ/cm^2; lane 2, 85 mJ/cm^2; lane 3, 45 mJ/cm^2; lane 4, starting oligonucleotide. The gel was electrophoresed at 1000 V for 2 hr, and the positions of the bands determined by autoradiography. The positions of inorganic phosphate and nucleotide were determined by running authentic sampl s in adjacent lanes.

The sample vaporized using a laser pow r of 45 mJ/cm^2 showed ext nsive strand scission, giving ris to

nucleic acid molecules having an average chain length of four nucleotides (Figure 11, lane 3; cf. starting sample in lane 4). Applicants believe that strand breakage occurred through activation of the phospodiester bond. Also, a great deal of the label was observed as free inorganic phosphate (P_i). However, the samples vaporized at 130 and 80 mJ/cm^2 (Figure 11, lanes 1 and 2) displayed no observable strand scission, although substantial amounts of phosphate were produced. There are several possible explanations for this observation. First, it is most likely that at lower laser powers the molecules remain in the thin film longer, causing the desorption process to mimic thermal decomposition, resulting in a much higher probability that bonds will be broken. Conversely, the vaporization process becomes a non-thermal, non-equilibrium photochemical process at high laser fluences. Second, the vaporized product should be less decomposed at high fluences because the density of desorbed material increases as the laser power increase. As the density increases, the number of cooling collisions increase and the vaporization process resembles a free jet expansion. A third possibility is that as the fluence of the vaporization beam increases, new electronic states in the desorbed rhodamine 6G are accessed via multiphoton absorption which serve to more efficiently transfer the nucleic acid molecules into the gas phase. This is supported by the fact that at the higher laser powers almost none of the laser dye travels to the filter paper without decomposition, whereas at lower incident fluxes much of the laser dye arrives intact.

Example 4

To more fully characterize the bond-breaking process observed in the 17-mer experiment, the vaporization of [α-^{32}P] dATP as a function of laser power was

studied. [α^{-32}-P] ATP (60 pmol, 3000 Ci/mmol) was dissolved in 20 μl f water containing 10% methan l and 10 mg/ml rhodamine 6G. Sample spots were prepared and vaporized as described above in Example 2 at powers equal to 320, 208, 180, 129, 85 and 45 mJ/cm^2 using a fresh spot for each power level. The resulting filters were processed as described above in Example 3, and the concentrated solutions adjusted so that each had 20,000 cpm/μl. Two microliters of each solution containing the materials which had been vaporized were then eluted from the filter papers, spotted onto a glass PEI-Cellulose F TLC plate (EM Science), and eluted with a solution of 0.6 M LiCl in 1.0 M formic acid. The plate was dried, and the amount of radioactivity present in each spot determined using an Ambis Radioactivity Image Scanner. The identity of the analyzed components was determined by co-spotting with authentic samples.

Referring to Figure 12, each point represents the average of at least two determinations from two TLC analyses. (O) dATP, (●) dAMP, (□) pyrophosphate, and (■) phosphate. This analysis indicates that up to five species are observed in the vaporized sample, depending on the vaporization condition. As predicted from the oligonucleotide experiments described in Example 3, the highest power levels result in little decomposition of the vaporized product. At 320 mJ/cm^2, approximately 90% of the radioactivity present on the filter paper was recovered as dATP. What little degradation that occurred resulted in the formation of dAMF and inorganic phosphates (mono-, diand tri-phosphates). As the laser power was reduced, several trends become evident: (i) less dATP survived the laser vaporization; (ii) less tripolyphosphate was observed (not shown; less than 4% was produced at ev n th highest pow r lev l); (iii) more phosphat and diphosphate were form d; and (iv) mor dAMP was produc d. Th pr ducti n f the in rganic

phosphate is consistent with photochemical activation of the P-O-P bonds at intermediate vaporization p wer. The incident ph ton fluenc was high enough that this activation proceeded through a two-photon excitation of the 274 num electronic absorption band in the phosphate group. At the highest powers, the P-O-P bonds were still activated, but the energy was most likely quenched by collisional deactivation. At low vaporization power, the desorption mechanism became more thermal in nature. As the molecules received thermal energy, the weakest bond, the phosphodiester bond, was expected to, and observed to, break.

Example 5

The fact that efficient molecular vaporization with very little bond breakage occurred at laser powers over 300 mJ/cm^2 suggested that it might be possible to effect the molecular vaporization of very long DNA strands. To test this prediction, two dideoxy DNA sequencing reactions were performed under conditions where the average chain length produced was either approximately 65 or approximately 400 nucleotides long. Two DNA sequencing reactions were carried out using SEQUENASE™ T7 DNA polymerase (United States Biochemical, Cleveland, OH) under conditions where the short (Figure 13, panel A) or long (Figure 13, panel B) DNA sequences were synthesized as described by the manufacturer.

For the reaction used to prepare short dideoxy C terminated DNA fragments (Figure 13, panel A), a 50 μl labeling reaction was prepared containing M13mp18 DNA, [α-^{32}P] dATP and Mn^{2+} buffer, using the manufacturer-recommended protocol. Immediately prior to vaporization, the DNA was d natur d and mix d with rhodamine 6G. This mixture (2μl) was spotted onto a glass cov r slip. The sampl was vaporized and the filters process d, as

described above in Examples 2 and 3. As was found in Example 2, with the vaporization of the nucleotide and oligonucleotide, the pattern of the autoradiograms revealed virtually no evidence for spallation (not shown). The ^{32}P-labeled materials were then eluted from the filters, and run on a high resolution polyacrylamide sequencing gel (Figure 13). The concentrated solutions were loaded onto a denaturing 8% polyacrylamide gel and electrophoresed for 2 hr at 55 watts: Figure 13, panel A, lane 1, 0.02 μl of the starting sequencing reaction prior to vaporization; panel A, lanes 2-5, samples recovered following vaporization at 320, 260, 210, and 160 mJ/cm^2, respectively.

The reaction used to prepare the long DNA sequences was virtually identical except that the Mn^{2+} buffer was not used and the termination mix contained a 3:2 ratio of normal dideoxy C termination mix and extension mix. Figure 13, Panel B, lanes 1-4, correspond to samples recovered following vaporization at 320, 260, 210, and 160 mJ/cm^2, respectively; panel B, lane 5 contained 0.006 μl of the starting sequencing reaction prior to vaporization. For both panels A and B, standard G, A, T, and C sequencing reactions were run in parallel in order to precisely determine the lengths of the indicated bands.

It is evident from this analysis that extremely large DNA molecules can be efficiently vaporized without any noticeable strand cleavage or degradation. In the case of the sequencing reaction containing products having an average length of 65 nucleotides (Figure 13A), bands up to 85 nucleotides in length were visible. Longer exposures (not shown) indicated the presence of longer strands (in the 120 to 140 nucleotide range). The banding pattern for the samples generated at each of the laser powers (Figure 13A, lanes 2-5) was as sharp as the starting material (Figure 13A, lane 1),

strongly suggesting that no strand degradation was occurring. However, unlike the 17-mer experiment, we cannot rule out the possibility of trace amounts of strand scission at random position on these long strands. The intensity distributions of the vaporized samples were substantially different than that of starting sample. For example, the ratios of the 20-mer to 75-mer were compared for the two samples by densitometric scanning of the autoradiograms. The results indicated that the intensity of the 75-mer bands were reduced in relative intensity by 90% for the vaporized sample. This latter point is further strong support for molecular vaporization, since spallation would be expected to generate materials on the filters having bands intensity distributions identical to the starting samples. Similar results are obtained from the sequencing reaction carried out to give very long labeled DNA strands (Figure 13B). Careful analysis of this gel revealed that DNA strands in excess of 1000 nucleotides long had been vaporized.

The sequencing method described here has numerous advantages over currently available or proposed approaches. First, it does not require that DNA sequencing products be run on a polyacrylamide gel. This component of manual or automated DNA sequencing is the most labor-intensive and time-consuming portion of DNA sequencing. Second, this method does not require the use of a radioisotope. Both this and the prior point should significantly reduce the expense currently associated with sequencing by reducing labor, chemical, and disposal costs. Also, the throughput that can be expected for this instrument may be 1000 times, or more, that obtained from currently available automated sequencers. An important consideration is that the technology described here can be easily automated for repeated sample analysis. Since the mass analysis can be ob-

tained very rapidly, a conservative estimate for th time involved in generating a single DNA s quence is c rtainly l ss than one minute, and sh uld be as little as a few seconds. Thus, an instrument running continuously might conceivably be able to sequence well above a million bases per day.

The apparatus described herein has not been used previously to make determinations of the molecular weights of biological samples. Because of the uniqueness of the apparatus, unique materials are prepared by virtue of the ionization occurring by the ionization laser after the DNA sample has been vaporized. It is because of this unique difference that the ionized molecules have enhanced capability of being detected more accurately and sensitively through the mass spec equipment. It is believed that these ionized molecules have not been described previously.

While the forms of the invention herein described constitute presently preferred embodiments, many others are possible. It is not intended herein to mention all of the possible equivalent forms or ramifications of the invention. It is understood that the terms used herein are merely descriptive rather than limiting, and that various changes may be made without departing from the spirit or scope of the invention. Other embodiments are within the following claims.

What Is Claimed Is:

1. A method analyzing an organic sample, comprising the steps of:

a) providing an organic sample in a medium which absorbs visible light;

b) vaporizing the sample and the medium into the gaseous phase by subjecting the sample and the medium to electromagnetic radiation from an optical source wherein the vaporizing of the organic sample occurs in a visible light absorbing medium which absorbs the electromagnetic radiation in the visible light region;

c) ionizing the vaporized sample; and

d) detecting the contents of the vaporized, ionized sample.

2. The method of claim 1 wherein the organic sample to be analyzed has an ionizable chromophore covalently bonded to the sample.

3. The method of claim 1 wherein the light absorbing medium is an organic dye.

4. The method of claim 1 wherein the light absorbing medium is in the liquid state.

5. The method of claim 1 wherein the light absorbing medium is in the solid state.

6. The method of claim 1 wherein the sample to be detected is selected from the group consisting of a saccharide, a polysaccharide, a lipid, a hormone, a neurotransmitter, a heme, a nucleotid , a nucl oside, a metabolite, and a peptide.

7. Th method of claim 1 wh rein th sample is a protein.

8. The method of claim 1 wherein the sample is a nucleic acid.

9. The method of claim 1 wherein the light absorbing medium has the ability to absorb light at a wavelength greater than 500 nanometers.

10. The method of claim 1 wherein the light absorbing medium is comprised of a dye.

11. The method of claim 2 wherein the chromophore to be bonded to the sample is a dye.

12. The method of claim 2 wherein the chromophore to be bonded to the sample is a fluorescent dye.

13. A method for analyzing a nucleic acid molecule comprising the step of vaporizing a mixture of said nucleic acid molecule and a matrix by illuminating said mixture with visible laser light absorbed by said matrix.

14. A method for determining the nucleotide sequence of a polynucleotide comprising the step of determining, by mass spectrometry, the relative molecular weights of single-stranded nucleic acid molecules in a population comprising a plurality of single-stranded nucleic acid molecules, each molecule having a different molecular weight and one defined terminus and one variable terminus, said variable terminus terminating at a specific nucl tide, wherein said m l cules ar gen rated from said polynucl otide.

15. The method of claim 14 wherein said determining step is performed without prior separation of each of said molecules from each other.

16. The method of claim 14 wherein said determining step comprises vaporizing a mixture of said population and said matrix by illuminating said mixture with visible laser light absorbed by said matrix.

17. The method of claim 14 wherein said nucleic acid molecules are generated by chemical degradation of said polynucleotide.

18. The method of claim 14 wherein said nucleic acid molecules are generated by extension of a primer, complementary to a portion of said polynucleotide, by a DNA polymerase in the presence of a chain terminating agent.

19. The method of claim 18 wherein said chain terminating agent is a dideoxynucleotide triphosphate.

20. The method of claim 18 wherein said DNA polymerase is T7 DNA polymerase.

21. The method of claim 18 wherein said extension is performed in the presence of manganese.

22. The method of claim 14 wherein said polynucleotide is DNA molecule.

23. The method of claim 14 wherein said polynucleotide is an RNA molecule.

24. The method of claim 14 wherein said determining step further comprises the step of comparing

molecular weights of said nucleic acid molecules g nerated fr m said polypeptid to provide said nucl otide sequence.

25. The method of claim 24 wherein said comparing step is performed using a computer.

26. The method of claim 1, 13 or 16 wherein said vaporizing step is performed using a laser adapted to emit a pulse of light.

27. The method of claim 26 wherein said laser is adapted to emit a pulse of light having a duration of less that 10 nanoseconds.

28. The method of claim 27 wherein the power of said laser light is greater than about 80 mJ/cm^2.

29. The method of claim 26 wherein said laser is a neodium yttrium aluminum garnet laser.

30. The method of claim 13 or 16 wherein said illumination is at a wavelength of from about 400 to about 1100 nm.

31. The method of claim 13 or 16 wherein said matrix is an organic dye.

32. The method of claim 31 wherein said organic dye is rhodamine 6G.

33. The method of claim 13 or 16 wherein said matrix is selected from the group consisting of Rhodamin 6G, Rhodamin 700 or 800, DTTCI, LC8800, DNTTCI, HDITCI, DDCI-4, and dib nz cyanine 45.

34. The method of claim 13 or 16 wherein said matrix has the ability to absorb light at a wavelength of from about 400 nm to about 1100 nm.

35. The method of claim 13 or 16 wherein said matrix absorbs light at a wavelength at which DNA does not absorb light.

36. The method of claim 13 or 16 wherein said matrix absorbs light at a wavelength at which RNA does not absorb light.

37. The method of claim 13 or 16 wherein said vaporizing step occurs without fragmenting said polynucleotide.

38. The method of claim 1, 13 or 16 wherein the molar ratio of said light absorbing matrix to said polynucleotide is from about 1:1 to about 100,000:1.

39. The method of claim 13 or 16 wherein a said nucleic acid molecule has a single positive charge.

40. The method of claim 13 or 16 wherein a said nucleic acid molecule is bound to an ionizable chromophore.

41. The method of claim 40 wherein a said nucleic acid molecule is chemically bound to said chromophore by a linker arm.

42. The method of claim 2 or 40 wherein said chromophore is selected from a group consisting of fluorescein, rhodamine, tetramethylrhodamine, sulforhodamine, nitrobenzyl-2-oxa-1-diazole, anthracen,

pyr ne, coumarin, acridone, N-5-dimethylaminonaphthene, and derivatives thereof.

43. The method of claim 42 wherein the derivatives are selected from the group consisting of iodoacetamide, maleimide, isothiocyanate and succinimidyl carboxylate.

44. The method of claim 13 or 16 further comprising the step of ionizing said nucleic acid molecules after said vaporizing step.

45. A method according to claim 1 or 44 wherein said ionizing is performed using a laser adapted to emitting a pulse of light.

46. The method of claim 45 wherein said laser is adapted to emit a pulse of light of less than 20 nanoseconds.

47. The method of claim 44 further comprising the step of determining the molecular weight of any vaporized, ionized nucleic acid molecule.

48. The method of claim 1 or 47 wherein said molecular weight is determined using a mass spectrometer.

49. The method of claim 48 wherein said mass spectrometer is a time-of-flight mass spectrometer.

50. The method of claim 14 wherein the molecular weights of said molecules in a plurality of said p pulati ns are d termined, said molecules in each said population having a variable terminus terminating

at a different specific nucleotide from other said populati ns.

51. The method of claim 50 wherein said method further includes comparing the molecular weights of said molecules with a computer.

52. A method for determining the base sequence of a nucleic acid strand wherein four separate nucleic acid samples derived from the strand are fragmented such that each fragment in a particular sample terminates at one of the bases adenosine (A), cytosine (C), guanosine (G), or thymidine (T), comprising the steps of:

(a) providing an organic sample comprised of the four samples of the nucleic acid in a light absorbing medium which absorbs light;

(b) vaporizing the sample and the light absorbing medium into the gaseous state;

(c) ionizing the vaporized sample; and

(d) detecting the contents of the vaporized, ionized sample.

53. An apparatus to analyze an organic sample present in a visible light absorbing medium comprising:

an optical source for generating electromagnetic radiation in the visible light region capable of vaporizing the organic sample in the visible light absorbing medium which absorbs visible light;

a source of ionizing radiation capable of ionizing the vaporized sample; and

an apparatus, juxtaposed to the vaporized ionized sample capable of determining the contents of the vaporized, ionized sample.

54. The apparatus of claim 53 wherein the apparatus capabl of determining the cont nts of th vaporized ionized sample is a mass sp ctrometer.

55. The apparatus of claim 53 wherein the source of vaporizing radiation is a laser.

56. The apparatus of claim 53 wherein the source of ionizing radiation is a laser.

57. The apparatus of claim 53 wherein the apparatus is capable of analyzing nucleic acid materials.

58. An apparatus for determining the nucleotide sequence of a polynucleotide, comprising:

(a) a mass spectrometer adapted to determine the relative molecular weights of individual single-stranded nucleic acid molecules in a first plurality of different populations comprising a second plurality of single-stranded nucleic acid molecules, each molecule having a different molecular weight, and one defined terminus and one variable terminus, said variable terminus terminating at a specific nucleotide, the variable terminus of each different first plurality of populations terminating at a different specific nucleotide, wherein said molecules are generated from said polynucleotide; and

(b) a computer adapted to compare the molecular weights of each of said molecules in said different first plurality of populations to provide the nucleic acid sequence of said polynucleotide.

59. The apparatus of claim 58 further comprising a las r adapted to generat light able t vaporiz said populations of molecules.

60. The apparatus of claim 58 furth r comprising a laser adapt d to generate light able to ionize said populati ns of mol cules.

61. A population comprising a plurality of single-stranded nucleic acid molecules, each molecule having a different molecular weight and one defined terminus and one variable terminus, said variabl terminus terminating at a specific nucleotide, said molecules being in an ionic and vapor form.

62. A plurality of populations of claim 61.

FIG. 1

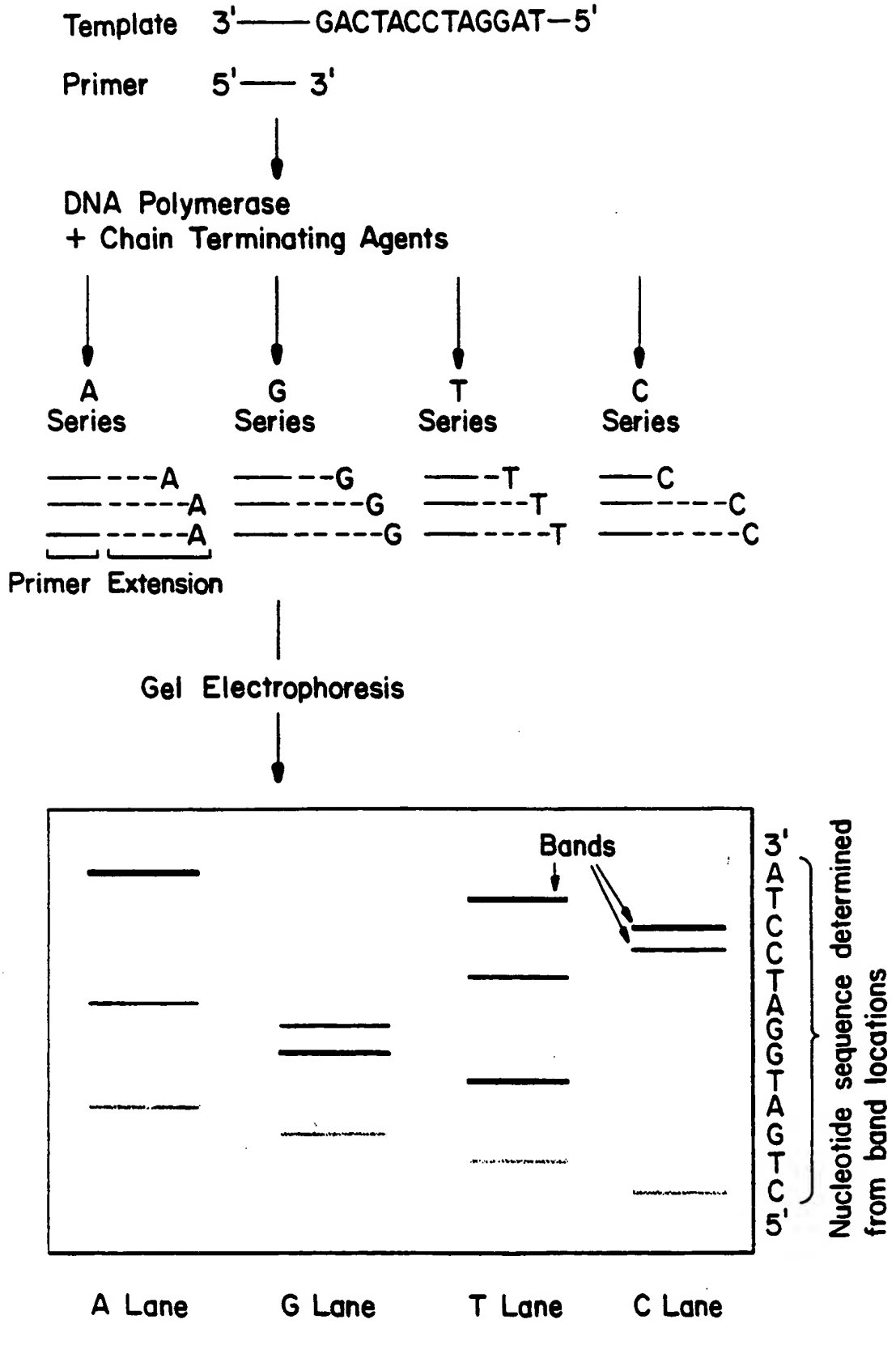

Template 3'——GACTACCTAGGAT—5'
Primer 5'—— 3'
DNA Polymerase
+ Chain Terminating Agents
A Series
G Series
T Series
C Series
Primer Extension
Gel Electrophoresis
Bands
3'ATCCTAGGTAGTC5'
Nucleotide sequence determined from band locations
A Lane
G Lane
T Lane
C Lane

FIG. 2

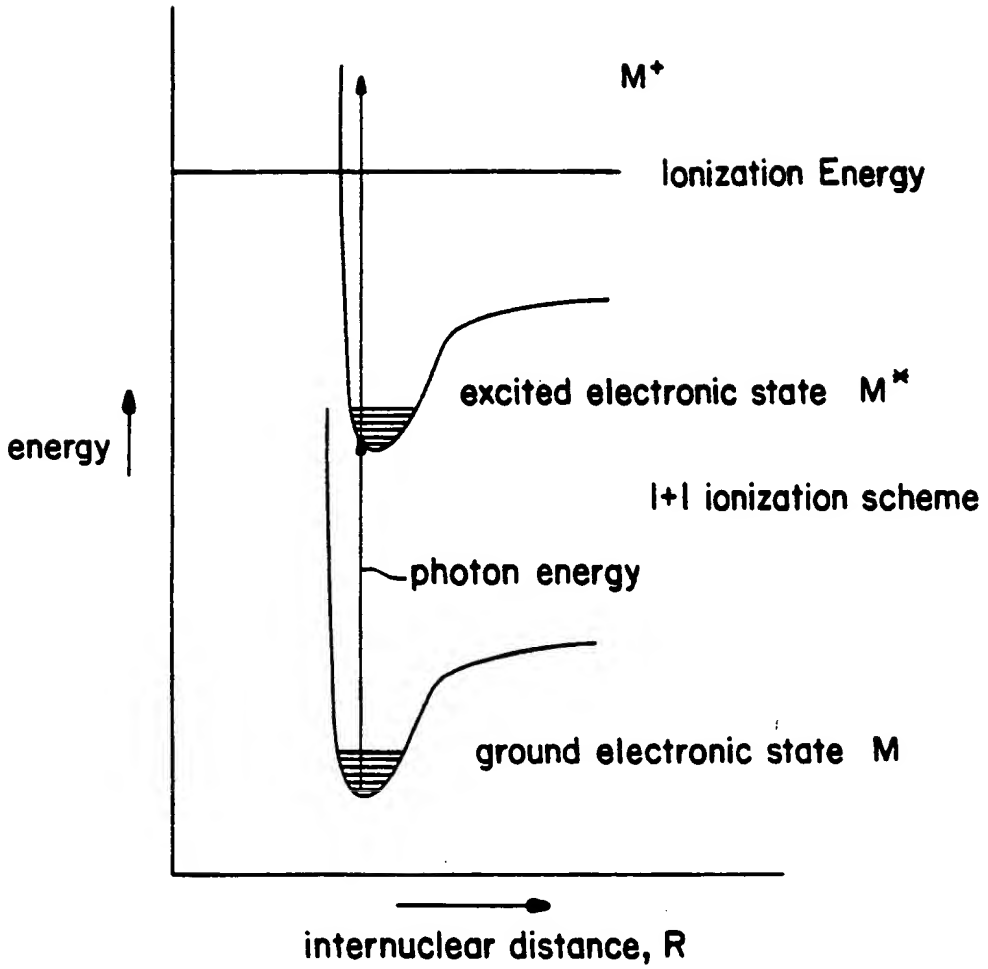
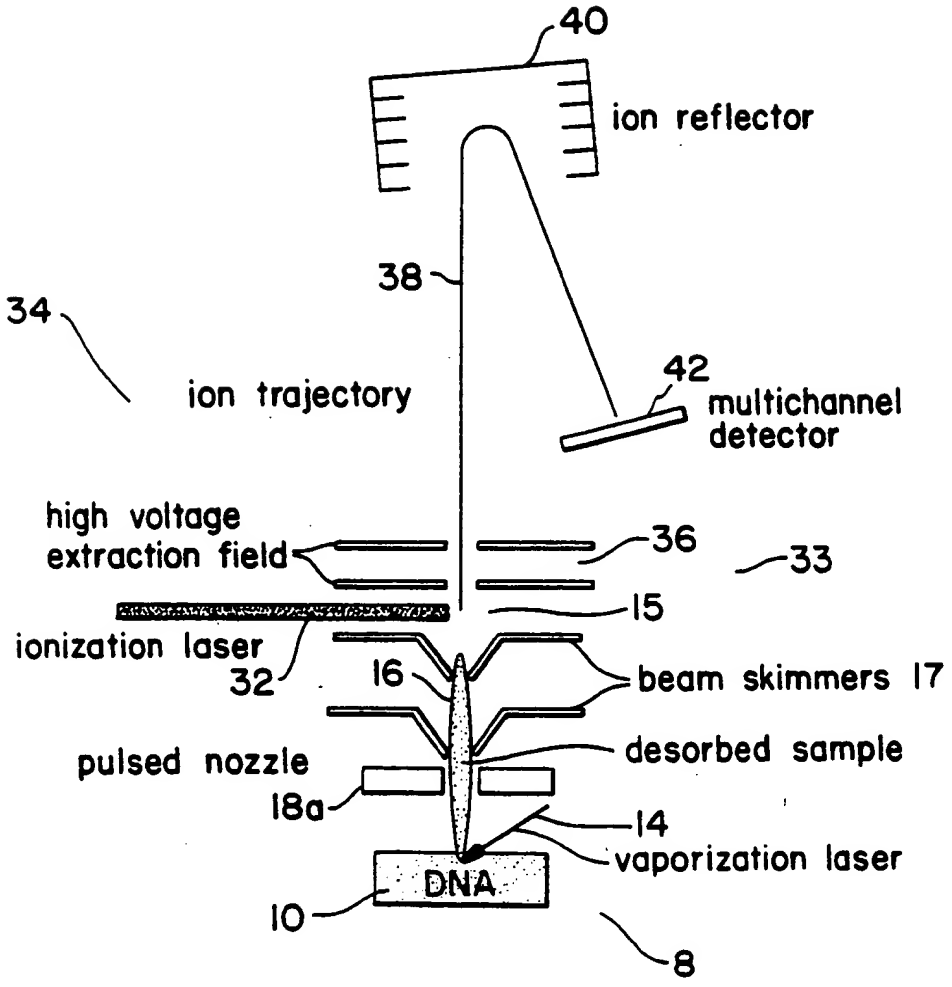

M+
Ionization Energy
excited electronic state M*
energy
I+I ionization scheme
photon energy
ground electronic state M
internuclear distance, R

FIG. 3


40
ion reflector
38
34
ion trajectory
42
multichannel detector
high voltage extraction field
36
33
15
ionization laser
32
16
beam skimmers 17
pulsed nozzle
desorbed sample
18a
14
vaporization laser
DNA
10
8

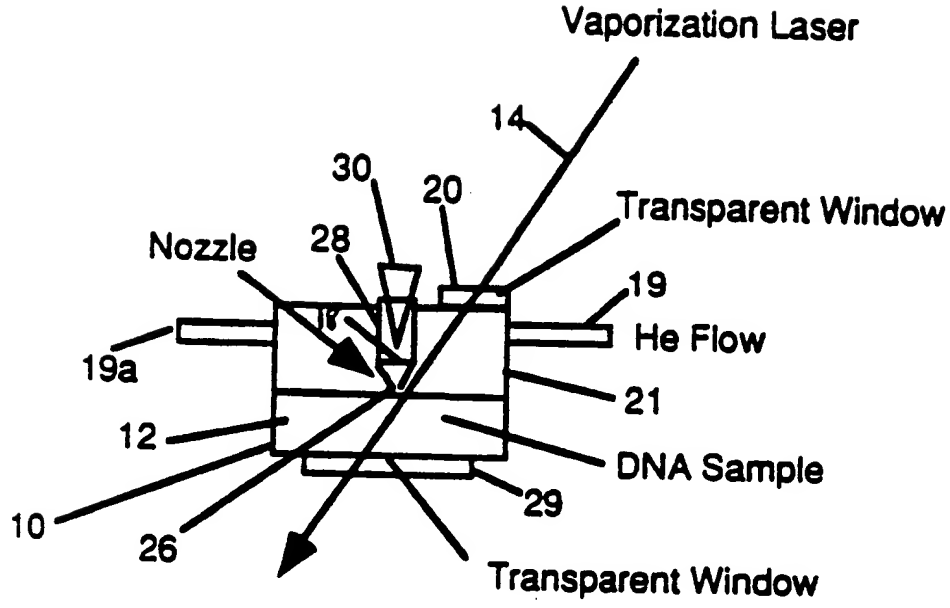

Vaporization Laser
14
30
20
Transparent Window
Nozzle
28
19
He Flow
19a
21
12
DNA Sample
29
10
26
Transparent Window

FIG. 4

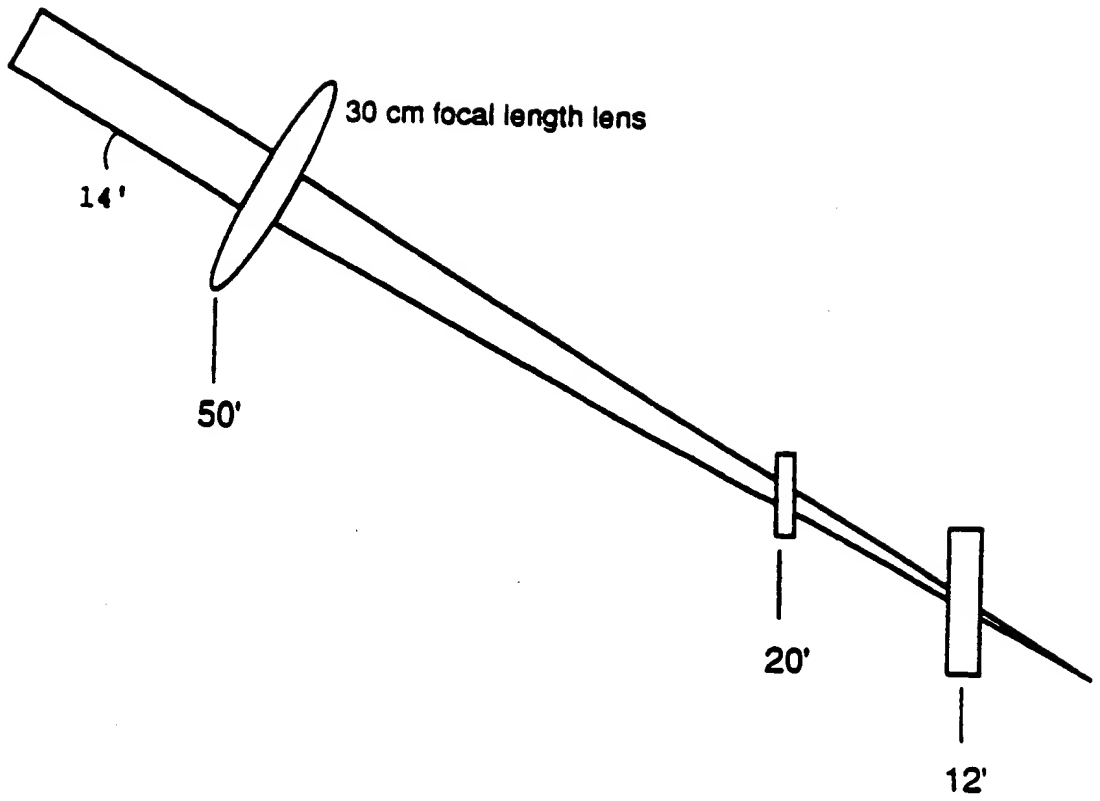


FIG. 5

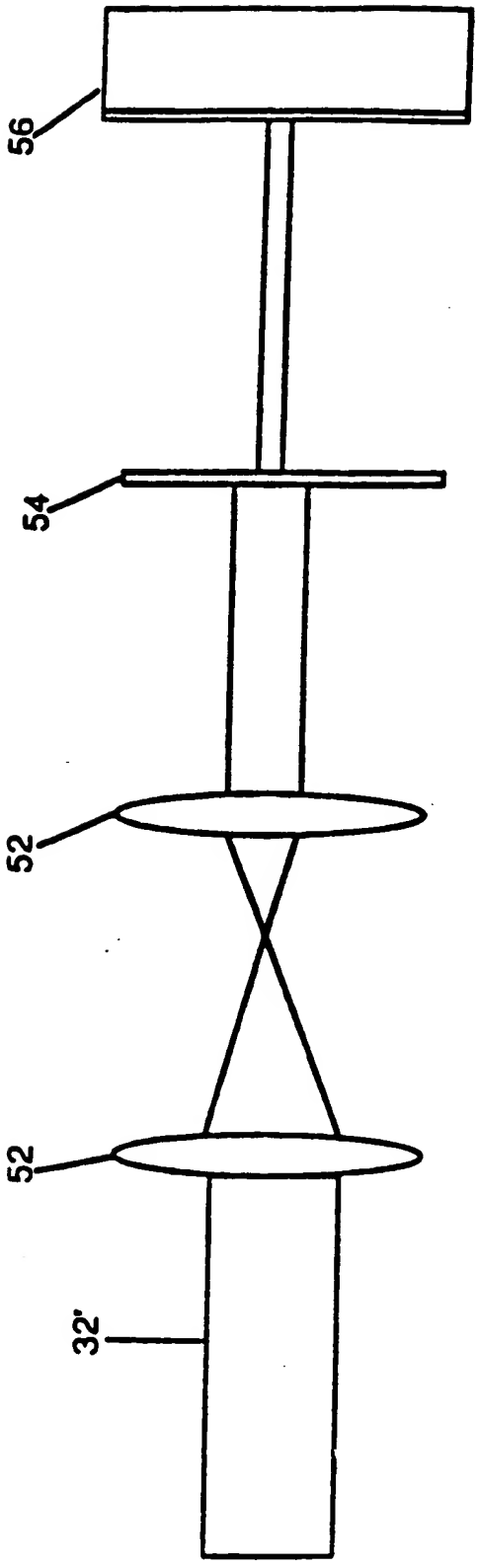

56
quartz window
54
iris
52
52
20 cm focal length telescope lense
S1 UV lenses
32'

FIG. 6

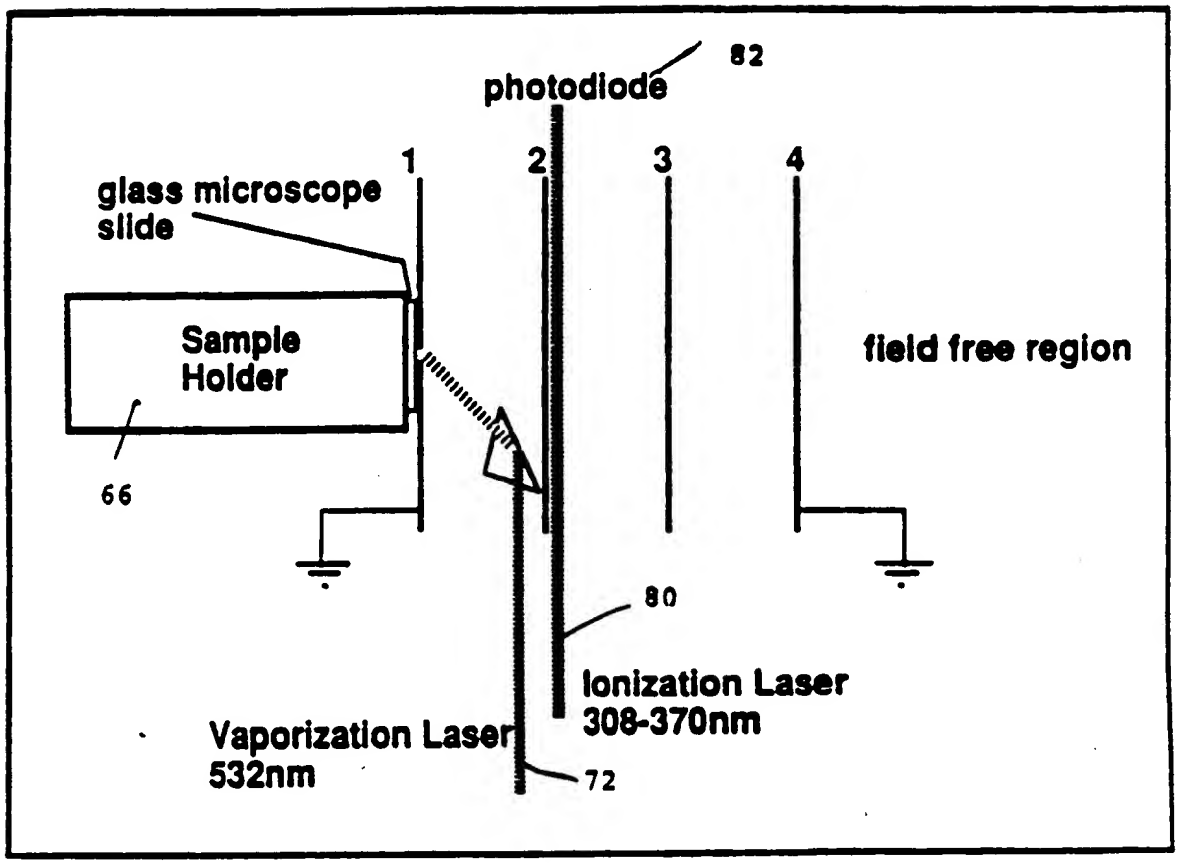

82
photodiode
1
2
3
4
glass microscope slide
Sample Holder
66
field free region
80
Ionization Laser 308-370nm
Vaporization Laser 532nm
72

FIG. 7

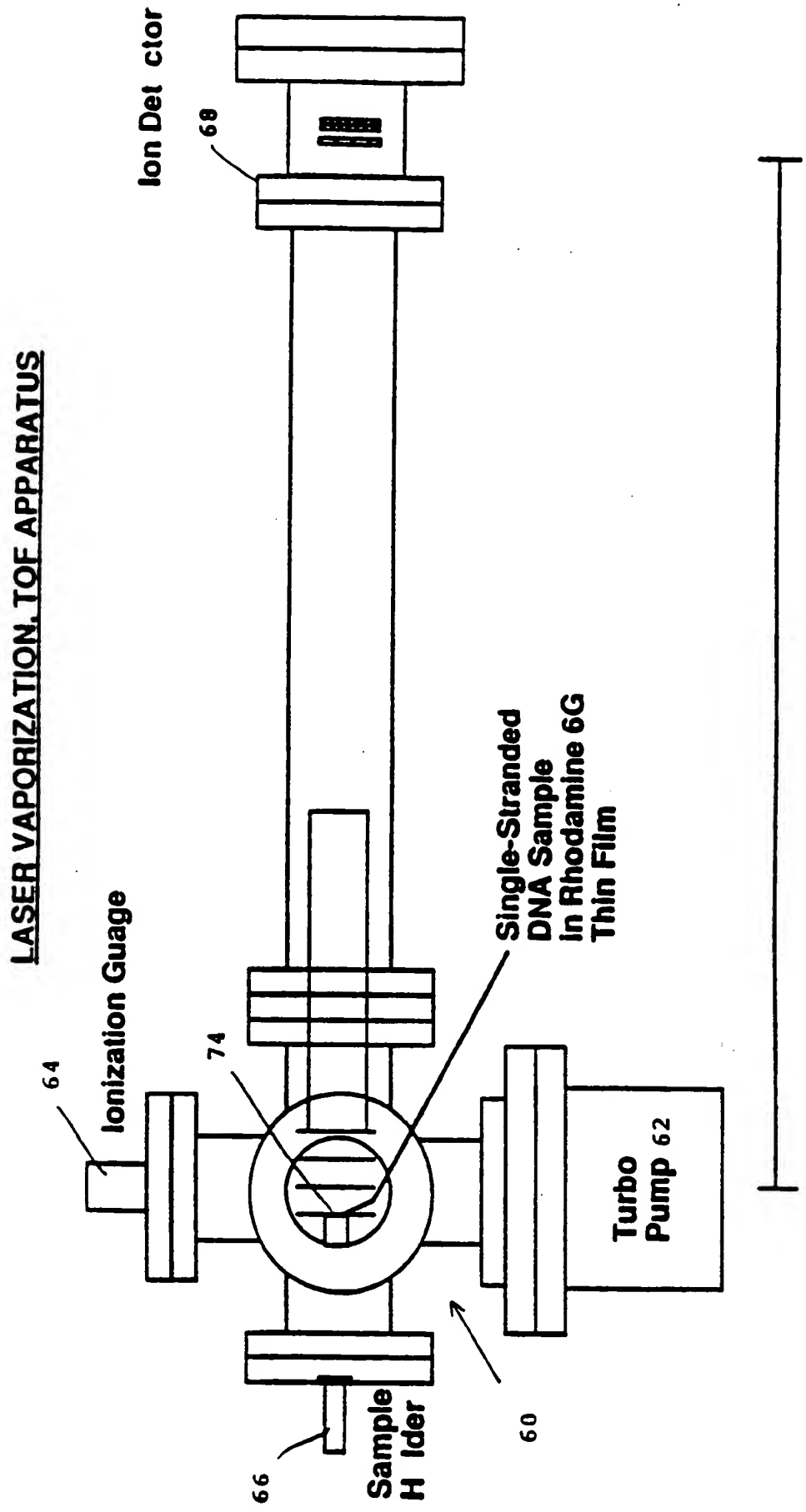

LASER VAPORIZATION TOF APPARATUS
Ion Det ctor
68
Ionization Guage
64
74
Single-Stranded
DNA Sample
in Rhodamine 6G
Thin Film
Turbo
Pump 62
63cm
Sample
H lder
66
60

FIG. 8

FIG. 9

Time-of-Flight Mass Spectrum of Antracene-linked Thymidine-5'-monophosphate

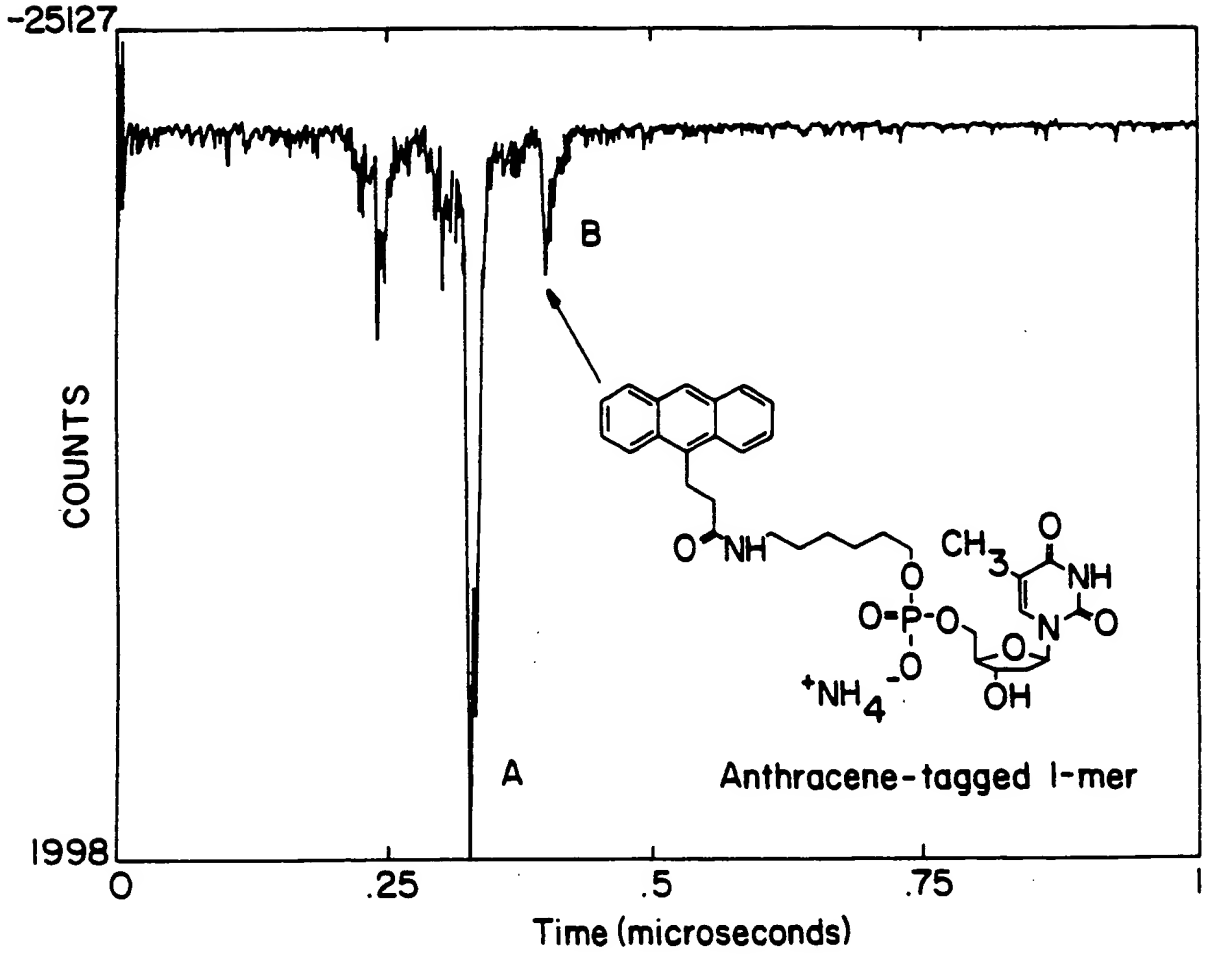


FIG. 10A

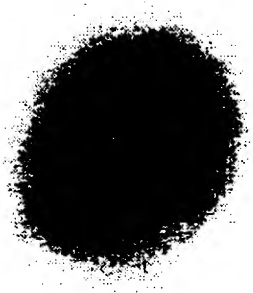


FIG. 10B

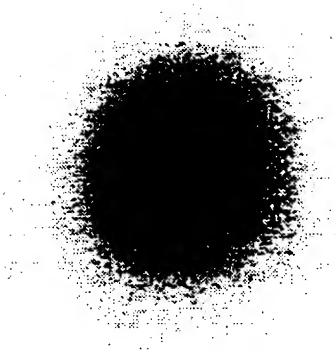


FIG. 10C

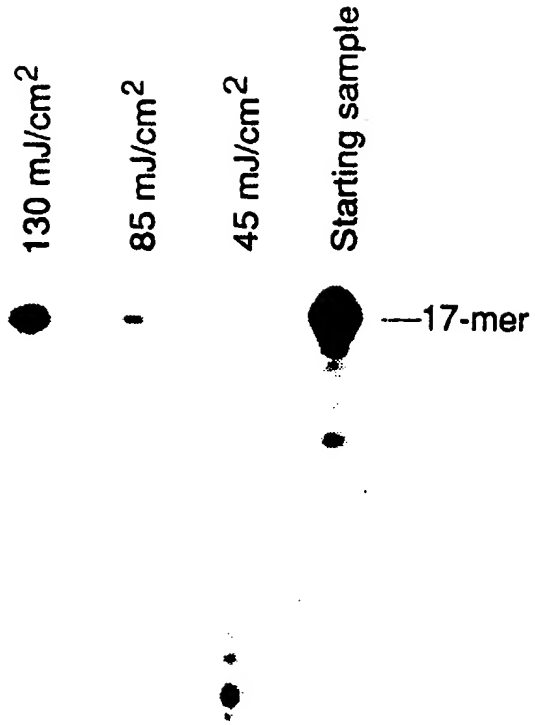

130 mJ/cm2
85 mJ/cm2
45 mJ/cm2
Starting sample
—17-mer
—1-mer
—Pi
1
2
3
4

FIG. 11

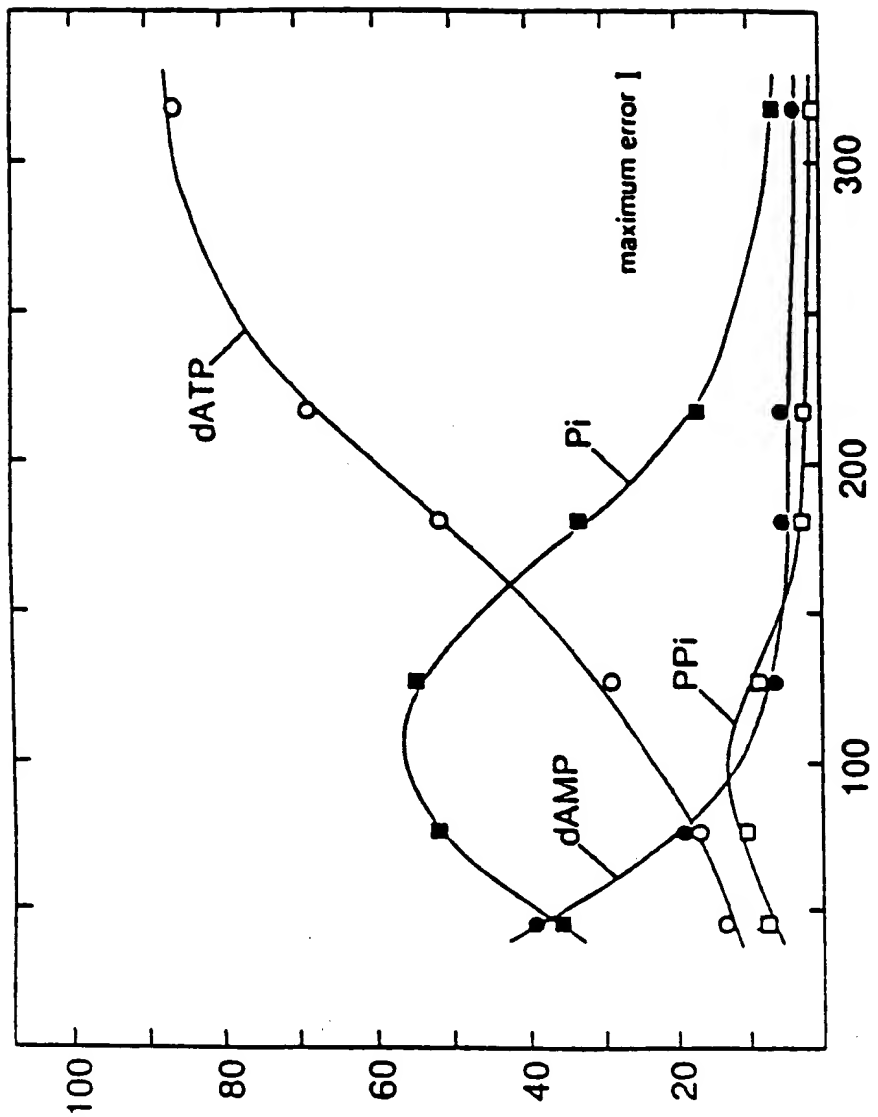

dATP
Pi
PPi
dAMP
maximum error
100
80
60
40
20
100
200
300
32P-Containing components recovered (%).
Laser power (mJ/cm2)

FIG. 12

FIG. 13A

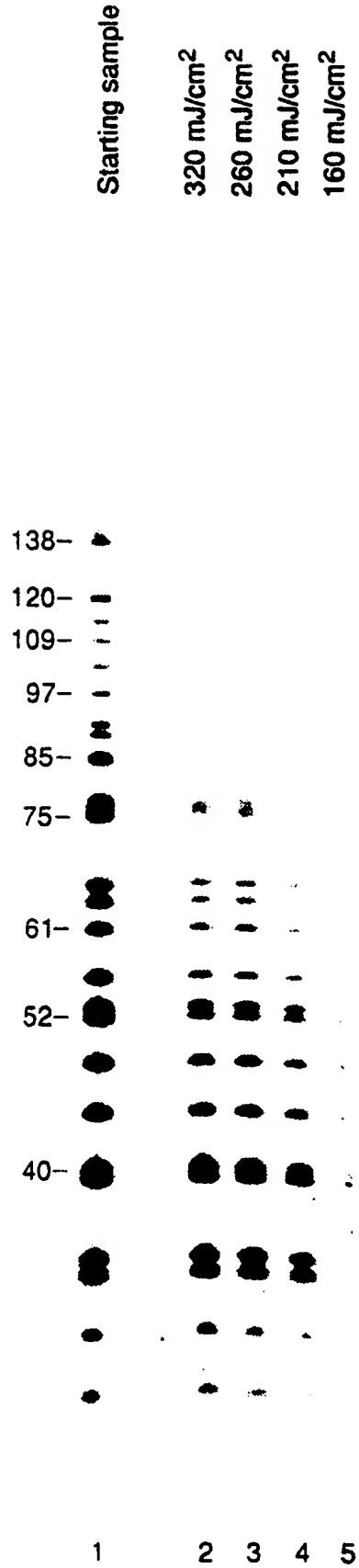
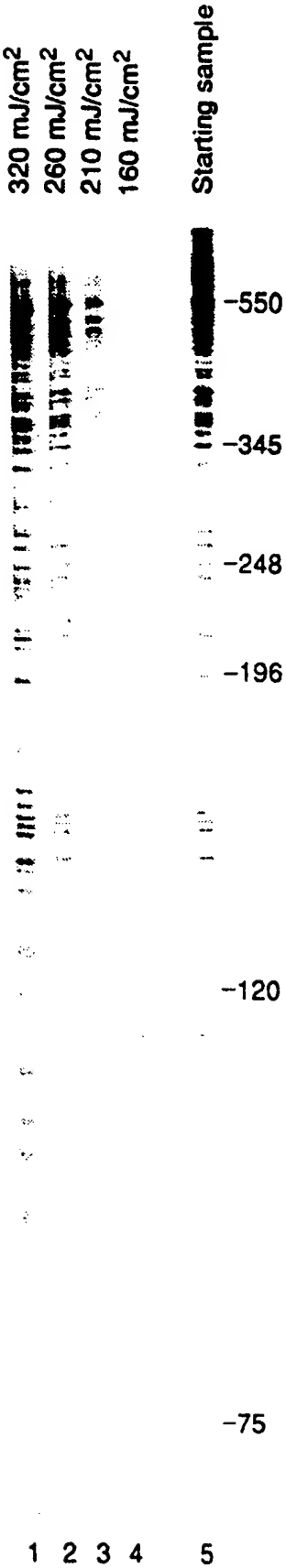


FIG. 13B



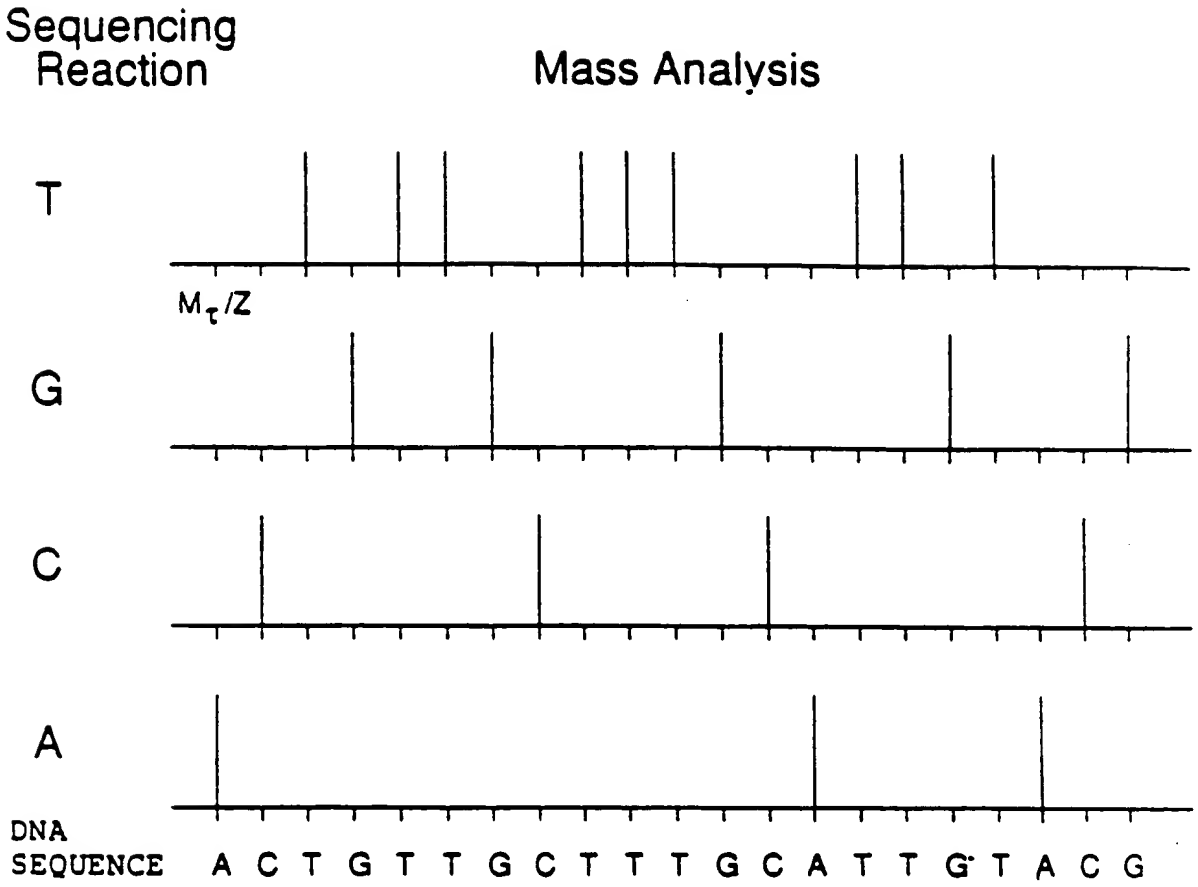

Sequencing Reaction
Mass Analysis
T
G
C
A
M_t/Z
DNA SEQUENCE
A C T G T T G C T T T G C A T T G T A C G

FIG. 14

INTERNATIONAL SEARCH REPORT

International Application No. PCT/US92/00714

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all) [6]

According to International Patent Classification (IPC) or to both National Classification and IPC

IPC(5): B01D 59/44, H01J 49/00 U.S. CL. 250/282,287,288

II FIELDS SEARCHED

Minimum Documentation Searched [7]

Classification System	Classification Symbols
U.S.	250/282,287,288,423P

Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in the Fields Searched [8]

III. DOCUMENTS CONSIDERED TO BE RELEVANT [9]

Category *	Citation of Document, [11] with indication, where appropriate, of the relevant passages [12]	Relevant to Claim No. [13]
X	US,A, 4,920,264 (BECKER) 24 April 1980, See Col 5, lines 13-47 and Fig 2.	1-5 45/1-46, 5-3-5-4.
X	US,A, 4,988,879 (ZARE ET AL) 29 January 1991, See Col. 7, lines 49-66.	6-9,26/1-29, 45/1-46, 48/1-49,53-57
X	Biomedical and Environment al Mass Spectrometry, Vol. 18, issued 1989, Karas et al., Ultraviolet - Laser Desurption/Ionization Mass Spectrometry of Femtomolar Amounts of Large Proteins, pp 841-843, See entire article.	10-12
A	US,A, 4,757,141 (FUNG ET AL) 12 July 1988, See entire documents.	
A	US,A, 4,855,225 (FUNG ET . AL) 08 August 1989, See entire document.	

* Special categories of cited documents: [10]

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

IV. CERTIFICATION

Date of the Actual Completion of the International Search	Date of Mailing of this International Search Report
08 May 1992	15 JUN 1992

International Searching Authority	Signature of Authorized Officer
ISA/US	NGUYEN TRUONG INTERNATIONAL DIVISION Bruce C. Anderson

Form PCT/ISA/210 (second sheet) (Rev. 11-87)